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THE EFFECT OF CERTAIN RESPIRATORY INHIBITORS ON THE RESPIRATION OF CHLORELLA.

By ROBERT EMERSON.

(From the Kaiser William Institute for Biology, Berlin-Dahlem, Germany.)

(Accepted for publication, December 2, 1926.)

INTRODUCTORY REMARKS.

This paper presents experiments on the effect of hydrocyanic acid, hydrogen sulfide, and carbon monoxide on the respiration of the green alga, *Chlorella*. The subject is of interest because all three of these substances are known to inhibit respiration specifically and reversibly in various organisms.

Hyman,[1] in a paper on the effects of potassium cyanide on *Planaria*, has reviewed the cases where direct measurements of the effects of cyanides on respiration have been made. Negelein[2] has published experiments on the effects of hydrogen sulfide, and Warburg[3] on the effects of carbon monoxide.

It has been shown in this laboratory that these three inhibitors of respiration do not check the respiration of *Chlorella*. Warburg[4] has shown this for hydrocyanic acid, Negelein[2] for hydrogen sulfide; I have tried the effect of carbon monoxide.

As will be shown, this exceptional behavior of *Chlorella* vanishes when the alga is made heterotrophic.

Methods.

The methods used in this work were essentially the same as those of Warburg and Negelein in their work on *Chlorella*. The alga was cultivated as described by them,[5] in a water thermostat lighted continuously with three 75-watt metal

[1] Hyman, L. H., *Am. J. Physiol.*, 1919, xlviii, 340.
[2] Negelein, E., *Biochem. Z.*, 1925, clxv, 203.
[3] Warburg, O., *Biochem. Z.*, 1926, clxxvii, 471.
[4] Warburg, O., *Biochem. Z.*, 1919, c, 268.
[5] Warburg, O., and Negelein, E., *Z. physik. Chem.*, 1922, cii, 250.

filament lamps, about 30 cm. distant from the culture flasks. A slow stream of 5 per cent carbon dioxide in air was bubbled through the cultures. They were pure for the most part, but in control experiments with cells from cultures where no precautions were taken to exclude bacteria the same results were obtained as with cells from cultures known to be pure. The amount of *Chlorella* cells used so far exceeded that of any chance bacteria, that the latter did not affect the results.

Respiration was measured manometrically, in the dark. Measured quantities of cell suspension were pipetted into vessels of the type shown in Fig. 1. The vessels were connected by gas-tight joints with their respective manometers. The gas space above the cells, and the capillaries, as far as the manometric fluid, were filled with a mixture of 5 volumes per cent carbon dioxide in air before the

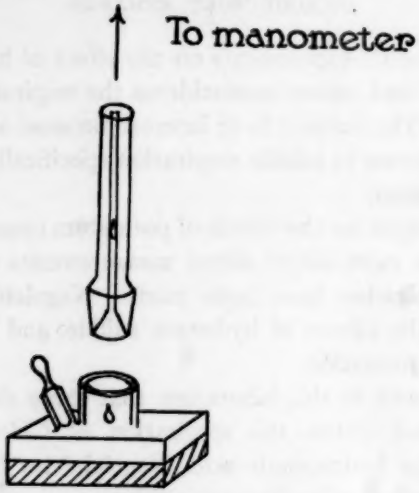

FIG. 1

system was closed. The vessels were shaken in a water thermostat at 20°C. in order to maintain a state of equilibrium between the gas in the space above the cell suspension and that dissolved in the suspension. It was always fast enough so that an increase caused no change in the results. At intervals the shaking was interrupted for the purpose of reading the manometers.

The principle involved here is that oxygen is less soluble in the cell suspension than carbon dioxide. Thus, if the cells, in respiration, consume oxygen and give off the same amount of carbon dioxide, the pressure in the gas chamber will decrease, causing a change in the manometer (*cf.* Warburg[6] for a detailed discussion of this method).

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[6] Warburg, O., *Biochem. Z.*, 1924, clii, 51.

Experiments with Hydrocyanic Acid.

The experiments with hydrocyanic acid will be described first and most completely. They are typical of those performed with hydrogen sulfide and carbon monoxide.

The concentration of hydrocyanic acid was 10^{-4} normal in practically all experiments. This is a convenient concentration for purposes of comparison, as Negelein has used it also. Its effect on

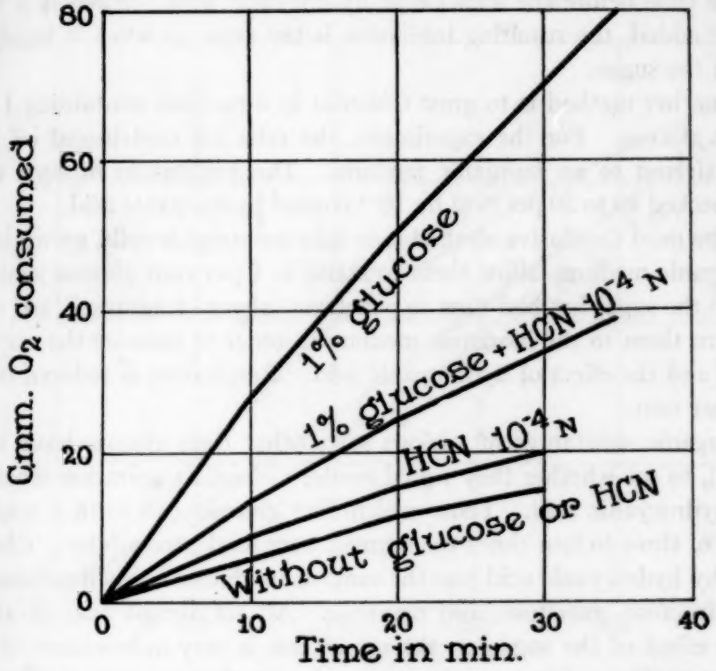

FIG. 2.

ordinary *Chlorella* cells (*i.e.* cells grown in an inorganic medium in bright light), is shown by the two lower curves of Fig. 2. Respiration is slightly accelerated. The two upper curves of Fig. 2 show the effect of the same concentration of hydrocyanic acid on the respiration of similar cells suspended in a solution containing 1 per cent glucose. It reduces their respiration over 50 per cent. Since all four curves represent the oxygen consumptions of the same amounts of cells, a com-

parison of the top and bottom curves shows that 1 per cent glucose about quadruples respiration.

Osterhout[7] and Krehan[8] have shown that hydrocyanic acid has definite effects on the permeability of living cells. Hence it might be argued that the acid merely checks the penetration of the sugar into the cells, and not the oxidation process inside them. It may be shown in various ways that this is not the case.

Cells may be allowed to remain in a medium containing sugar for some time before the addition of hydrocyanic acid. When it is thus later added, the resulting inhibition is the same as when it is added with the sugar.

Another method is to grow *Chlorella* in a medium containing 1 per cent glucose. For the experiment, the cells are centrifuged off and transferred to an inorganic medium. The respiration of such cells is checked 40 to 50 per cent by 10^{-4} normal hydrocyanic acid.

The most conclusive method is to take autotrophic cells, grown in an inorganic medium, allow them to stand in 1 per cent glucose solution until the sugar has had time to penetrate (about 15 minutes), and then return them to the inorganic medium in order to measure the respiration and the effect of hydrocyanic acid. Respiration is reduced 60 to 70 per cent.

Organic substances of various sorts other than glucose have been tried, to see whether they would render *Chlorella* respiration sensitive to hydrocyanic acid. Those which, like glucose, call forth a respiration of three to four times the normal, were used successfully. Checking by hydrocyanic acid was the same as in glucose. Such substances are fructose, galactose, and mannose. All act almost exactly alike. The effect of the sugar on the respiration is very independent of the concentration. Glucose, for example, accelerates the respiration about four times, whether it is present in 4/100 per cent or 4 per cent solution. The inhibition by hydrocyanic acid is likewise independent of the sugar concentration.

Other substances tried gave slight acceleration of respiration (in general less than double). Many indifferent substances will cause this slight acceleration. But no real sensitivity to hydrocyanic acid

[7] Osterhout, W. J. V., *Bot. Gaz.*, 1917, lxiii, 77.

[8] Krehan, M., *Internat. Z. physik.-chem. Biol.*, 1914, i, 189.

results. Although respiration may be checked slightly at first, it is soon accelerated, just as with ordinary cells. The substances tried were cane-sugar, arabinose, dioxyacetone, glycocoll, mannitol, and lactic acid. Fig. 3 shows the effect of hydrocyanic acid on cells suspended in a solution containing 1 per cent glycocoll. Glycocoll was selected as typical of the indifferent substances.

Various authors (*e.g.* Hyman[1]) have stressed the reversibility of the hydrocyanic acid inhibition. If the effect be irreversible, it is argued

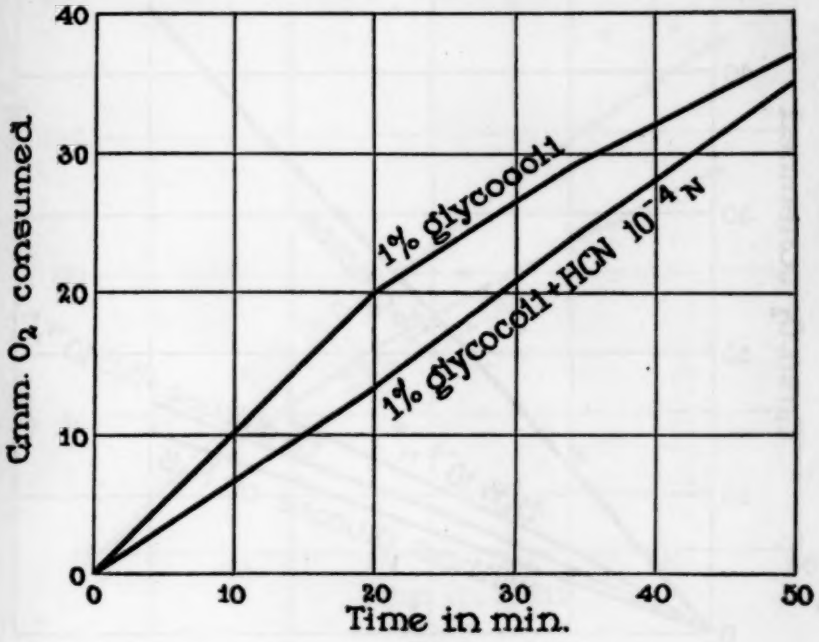

FIG. 3.

that the cells have been injured, and any inhibition of respiration cannot be regarded as specific. To test the reversibility in the case of sugar-containing *Chlorella* cells, the cells were subjected to the effect of hydrocyanic acid for 30 minutes, the checking of respiration measured, and then a stream of moist air was bubbled through the suspension, to remove the hydrocyanic acid. Respiration was then measured again, and found to be greater than in the control. The effect is therefore completely reversible.

Experiments with Hydrogen Sulfide.

Similar experiments were carried out with hydrogen sulfide and led to the same results as with hydrocyanic acid. Free hydrogen sulfide was formed in the cell suspension by the addition of an appropriate amount of N/10 solution of sodium sulfide. The solution

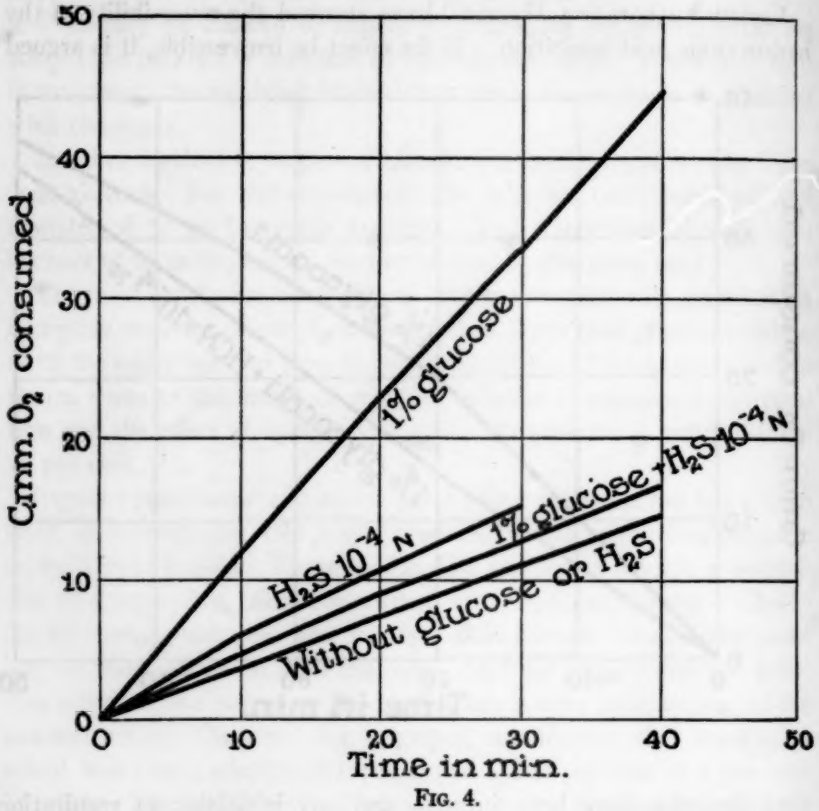

FIG. 4.

always contained acid phosphate which reacts with Na_2S to form free H_2S. (For the calculation of the correct amount of sodium sulfide to add in order to achieve a given concentration of hydrogen sulfide in the solution, see Negelein's paper on the effects of hydrogen sulfide.) Equilibrium between solution and the gas chamber is established, a large amount of hydrogen sulfide remaining in the gas chamber.

Fig. 4 shows the effect of hydrogen sulfide on ordinary *Chlorella* cells and on cells suspended in 1 per cent glucose solution. It is essentially the same as Fig. 1, which shows the corresponding curves for hydrocyanic acid.

The hydrogen sulfide effect was tested for reversibility, and it was found, like that of hydrocyanic acid, to be completely reversible. After removal of the hydrogen sulfide, respiration was greater than before it had been added.

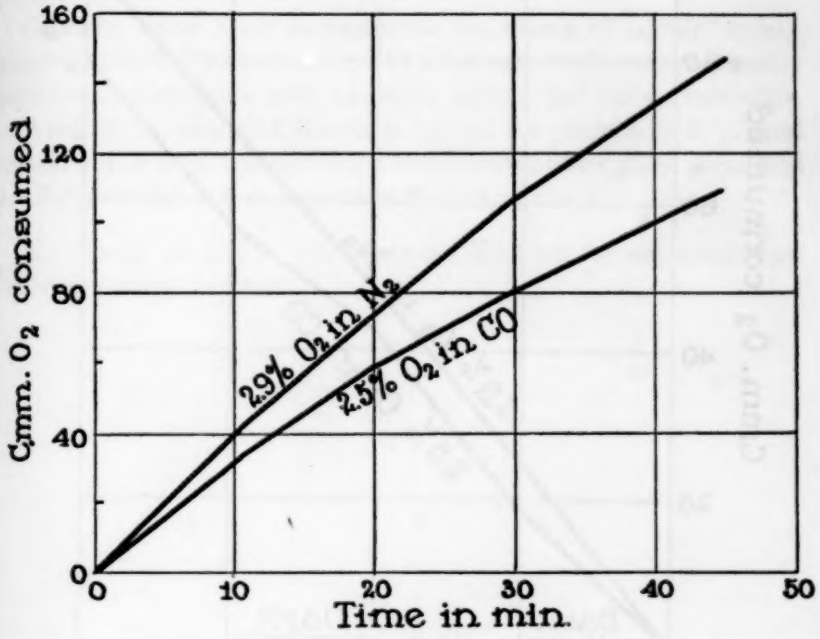

FIG. 5.

Experiments with Carbon Monoxide.

In the experiments with carbon monoxide, a mixture of oxygen and carbon monoxide replaced the usual 5 per cent carbon dioxide in air used in the gas space. As control the same amount of oxygen in nitrogen was used. Fig. 5 shows the effect of a mixture of approximately 2.5 volumes per cent oxygen in carbon monoxide on *Chlorella* suspended in a solution containing 1 per cent glucose. No curves are

given for the effect of carbon monoxide on ordinary *Chlorella* cells, for it does not affect their respiration.

The inhibition of respiration by carbon monoxide is completely reversible. Carbon monoxide was removed by passing the oxygen-nitrogen mixture through the gas chamber.

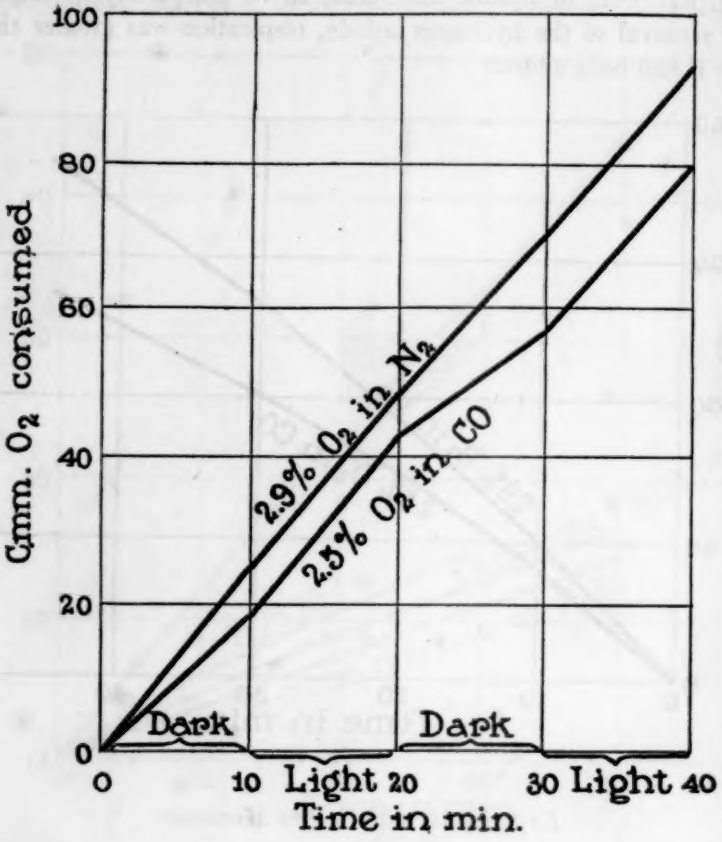

FIG. 6.

Warburg[3] has shown that the inhibition of yeast respiration by carbon monoxide practically vanishes in light. This experiment cannot be performed with ordinary *Chlorella*, as photosynthesis takes place in the light. But yellow non-photosynthesizing *Chlorella*, practically chlorophyll-free, may be produced in a medium containing

1 per cent glucose and of low iron content. Such cells are completely heterotrophic, and their respiration is inhibited by hydrocyanic acid and hydrogen sulfide. Respiration is practically the same in light as in darkness. Fig. 6 shows the effect of successive periods of light and darkness on the respiration of such cells in 2.5 per cent oxygen in carbon monoxide, and in 2.9 per cent oxygen in nitrogen.

SUMMARY.

Chlorella, when made heterotrophic by means of certain sugars, respires like other heterotrophic cells when subjected to the respiratory inhibitors, hydrocyanic acid, hydrogen sulfide, and carbon monoxide.

Whether the case of *Chlorella* is typical for green cells in general remains to be seen. Experiments with various other green organisms are being carried out, in hope of settling this point.

My thanks are due to Professor Otto Warburg for suggestion and criticism during this work.

THERMAL INCREMENTS FOR PULSATION-FREQUENCY IN "ACCESSORY HEARTS" OF NOTONECTA.

By W. J. CROZIER and T. J. B. STIER.

(*From the Laboratory of General Physiology, Harvard University, Cambridge.*)

(Accepted for publication, January 20, 1927.)

I.

For development of a theory of the temperature characteristics of vital processes it is necessary to deal with types of material in which it may be expected that experimental treatments can evoke one or another of a series of interrelated critical increments (Crozier, 1924–25, *a*, *b*; Crozier and Stier, 1924–25, *a*; 1925–26, *b*). In this paper we give an initial account of temperature relations in a kind of rhythmic activity which is in certain respects especially favorable for such tests.

Pulsatile organs, "accessory hearts," were first observed in the legs of juvenile aquatic hemiptera (*Notonecta*, *Ranatra*, *Corixa*, and others) by Behn (1835). These organs, located generally in the tibia just distal to the femoral articulation, or in the tarsus, were conceived by Behn to be a kind of "membranous valve" important in maintaining the peripheral circulation. Their "spontaneous" rhythmic movements were apparently confirmed, although misinterpreted, by Dufour (1835); they were discussed by Dugès (1838[1]), and by Verloren (1847[2]), and a summary of the early observations on the "accessory hearts" in aquatic and other hemiptera was given by Edwards (1858). It was noted (Verloren, 1847) that in *Tettigonia* the pulsatile activity might be interrupted for more or less lengthy periods, but that when active the frequency of the quick, sharp contractions exceeded that of the heart. The "hearts" were rediscovered by Mitchell (1858). Locy (1884) also described their movements, in *Ranatra*, *Notonecta*, and *Bdelostoma*, subscribing to their rôle in connection with the circulation; he noted that the movements would continue for a time in amputated legs, and even when the "heart" was itself cut into parts. More recent

[1] Dugès, 1838, p. 441.

[2] Verloren, 1847, p. 82.

observations, with figures, are given by Brôcher (1909). The existence of these organs is briefly referred to in entomological texts (Houlbert, 1920; Schröder, 1913), but nothing further appears to be known about these curiously inviting instances of perhaps myogenic rhythm. Among a number of possible sources of pulsatile organs in the bodies of insects (*cf.*, *e.g.*, Brôcher, 1916; 1917; 1919) they are by far the most amenable to experimental manipulation. They can be observed in the intact animal, and proper technique provides preparations in which the organs are active for days in detached legs. Six preparations may be gotten from a single individual, permitting for some purposes unusual control material.

In our experience the locomotor muscles of amputated legs of *Corixa* are likely to exhibit twitching movements, which interfere with the activities of the "accessory hearts," and in *Ranatra* the latter are difficult to see. Common species of *Notonecta*, however, are obtainable in large numbers, live very well in the laboratory, the "hearts" are easily seen, and the limb muscles of the detached legs are absolutely quiescent. The following account is based entirely upon the behavior of "accessory hearts" in the two swimming-legs of adult *Notonecta undulata*. It will be shown that although two preparations are obtainable from each individual these may nevertheless behave in quantitatively different ways, so that perfect control observations are not possible. But no necessity for such controls arose in the present work, which was designed to obtain primarily the temperature characteristics for pulsation-frequency during the survival of the isolated limb.

Our purpose was to discover if in different freshly isolated legs, and during the course of the irreversible death phenomena, there should not appear, as in the breathing movements of grasshoppers (Crozier and Stier, 1924–25, *a*), a series of differing critical thermal increments for pulsation-frequency, which might thus reveal certain of the interrelated processes involved in determining the rate of pulsation.

We were especially anxious to do this because of the possibility of obtaining information about typical governing processes in insect muscle, for comparison with central nervous activities (Crozier, 1924–25, *a*; Crozier and Federighi, 1924–25, *a*, *b*; Crozier and Stier, 1924–25, *a*, *b*; Fries, 1926–27). Although possibly containing nervous elements, the "hearts" in isolated legs are obviously beyond central nervous influence. It turns out that the typical temperature char-

acteristics obtained for frequency of pulsation agree quantitatively with those known for respiratory phenomena (Crozier, 1924–25, *b*), and for breathing movements of insects (Crozier and Stier, 1924–25, *a*), but with the addition of a further member of this "set" of critical increments. They differ sharply from the value (μ = 12,300) which is characteristic for frequency of heart contractions in arthropods (Crozier, 1924–25, *a*; Crozier and Federighi, 1924–25, *a*; Crozier and Stier, 1925–26, *b*; Fries, 1926–27). The associations observed between the four chief increments obtained are of additional interest for the analysis of vital processes from this standpoint.

II.

The beating of the leg-"hearts" is easily observed in the intact animal. The back-swimming habit of *Notonecta* makes it possible to prepare an individual by mounting it, ventral surface upward, upon a small block of hard rubber, to which it is attached by a small amount of vaseline, in such a way as to be at the surface of a vessel of water, with the legs outstretched in the surface film. Occasionally, movements of the appendages are seen; but the intervals between these more or less periodic movements are fairly long. The rate of pulsation in any one leg is very uniform; thus in one case, observed at frequent intervals over 24 hours, at 17°C., the time for ten contractions varied between 9.6 and 1.06 seconds; in another, between 5.2 and 5.6 seconds during 36 hours; frequently there is some decline in rate after 12 hours.

The beats are irregular for brief periods; sometimes a contraction is skipped. There is no evidence of diurnal rhythm. When the two swimming-legs of one individual are compared, there is usually a distinct difference between them:

Animal No.	Leg	Time for ten pulsations, seconds								
N1	Left	5.4	5.6	5.3	5.5	5.5				
19°	Right	7.1	6.7	7.3	7.3	7.8	8.0			
N2	Left	5.0	5.1	5.1	4.7	4.1	5.2	4.9	5.8	5.0
19°	Right	4.4	4.9	4.7	5.6	5.1	5.5	5.7	4.4	
N3	Left	8.3	7.9	7.9	8.3					
17°	Right	7.4	7.0	7.6	7.0					

One leg-"heart" may abruptly cease operations for a time, the other continuing without pause. When the animal is stimulated, the beat may be hastened or retarded and irregularities appear.

The uncorrelated nature of the rates of contraction in the two legs and their independent variation speak for local rather than central nervous control of the activities of the leg-"hearts." Yet when the intact animal dies the frequency of the pulsations changes and to some extent their character also. When an animal in which the "heart" of one leg has been pulsating for some time at a steady frequency of 10.5 seconds for ten beats is completely covered by a thick layer of vaseline the rate remains constant for a time; but then, after several hours, the rate *suddenly* decreases to about one-half its former value, in both legs, and at this point the animal as a whole ceases to respond to stimulation. If left attached to the body the leg-"hearts" continue to beat for some 14 hours, but with decreasing although regular frequency. If detached from the body the legs show sustained pulsation for a much longer time. If the attached or isolated limb be swathed in vaseline no change in pulsation rate occurs.

These and other observations show that the rate of pulsation is locally determined, but that a secondary control, of nervous character, possibly, or dependent upon changes in the hemolymph pressure, is also important.

Two general types of result follow amputation of a swimming-leg. The rate of pulsation of the "heart" may remain the same as before amputation, the beat becoming at once more regular, and then, after about an hour (18°), the beat suddenly becomes much slower. In other cases the rate immediately after amputation may be about doubled, for several minutes, followed by sharp cessation of contraction which lasts about an hour; contractions are then resumed at about one-quarter of the original frequency. At the moment of pinching the leg with scissors the beats slow down, then become very rapid, then settle down to the initial rate. We have plugged the ends of amputated legs by exposing them to the air for 5 minutes, then by coating with vaseline; or the leg may be cut while imbedded in vaseline. No effects of these procedures were detectable.

It is important that the nature of the pulsatile movement of the leg-"heart" retains a very uniform character throughout the tempera-

ture range. Contraction is abrupt, relaxation slower, followed by an interval of quiescence. The contraction is maximal and always complete. The sheet of muscle fibers constituting the "heart" (*cf.* Brôcher, 1909) is cap-shaped at the proximal end, from which a (?muscle) band passes to the femorotibial joint; distally, the "cap" spreads out into a flat contractile band. As a rule, contraction first appears at the apex of the "cap," and travels as a wave to the distal band. Occasionally the contraction wave is reversed. As death approaches the relaxation phase is prolonged, and the quiescent interval almost disappears; this also happens if the leg is sealed in a tube of water previously boiled.

III.

For observation, the isolated legs of *Notonecta* were attached by vaseline to a glass slide placed in a small glass vessel of water. This vessel, having a flat bottom, was sunk in a chamber filled with water. The chamber was water-tight, and addition of hot or cold water for regulation of temperature was by way of a coil of copper tube, with many small apertures, connected with an external supply. This chamber was immersed in a large stirred thermostat. Through the cover of the vessel containing the preparations the objective of the observing microscope passed, and also a thermometer reading to 0.01°. The objective was used as a water-immersion lens, thus avoiding troublesome fogging, all the metal surfaces being coated with paraffin to obviate oligodynamic effects. The regulation of the temperature of the innermost vessel was secured by maintaining very slight differences between the temperature of the large thermostat and of the chamber immediately surrounding the observing vessel. Two, three, or four legs could be mounted together, and by adjustment of the microscope they could be viewed in succession or as desired. Light was reflected up through the thermostat and through a window in the inner box.

The muscles of an isolated leg, including the contractile fibers of its "heart," must of course be regarded as a system proceeding toward death, irreversibly. Therefore, if the relationship is to be obtained which exists between temperature and frequency of pulsation it is necessary to work rapidly in order to secure observations at a number

of temperatures before the underlying mechanism shall have changed materially. With these preparations it is possible to do so, largely because the latitude of variation in frequency of pulsations at constant temperature is so very slight.

After removal from the animal about 1 hour (at room temperature) is often necessary before the isolated "accessory heart" settles down to a steady rate of contraction. Not infrequently, however, this steady rate, lower than before amputation of the leg, is attained almost at once. A few successive readings of time for ten pulsations enable one to judge if the preparation is in a state suitable for experimentation. In order to discover the degree of constancy to be expected under uniform temperature, graphical records were obtained with the aid of a signal key and a chronoscope registering 1.0 second intervals upon a smoked drum. In this way a sufficiently precise record was obtained of continuous series of pulsations over periods of about ½ hour. The high degree of constancy in "time for ten pulsations" in any one preparation is shown in the following table:

Temperature	Preparation	Mean time for ten contractions
°C.		sec.
16.4°	A_1	19.0 ±0.4
16.4°	B_2	21.6 ±0.2
16.4°	E_2	19.2 ±0.3
16.1°	I	17.95±0.95
16.2°	K	15.7 ±0.4

In this table the ± limits give the *maximum* latitudes of variation, which average 4.9 per cent of the means. The same type of variation is evident in observations made at intervals, with a stop-watch, over periods of some hours. The maximum latitude of variation for any one heart is not over 10 per cent, and is as a rule less than 5 per cent of the mean at a given temperature. This variation of course includes errors of observation. The same type of fluctuation is apparent throughout the temperature graphs. In good preparations constancy in pulsation rate is maintained for many hours, at uniform temperature.

IV.

When the temperature is varied it is quite necessary to study each preparation individually. Averaging observations from different "accessory hearts" is not permissible. Even the two swimming-legs from the same individul may differ significantly (Figs. 7, 8), although they not infrequently give parallel results (Figs. 1 to 5).

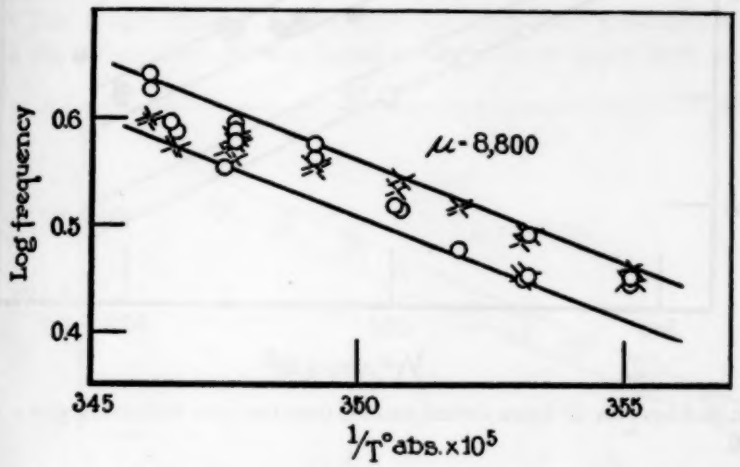

FIG. 1. In this and succeeding figures the temperature characteristics for frequency of pulsation in the accessory leg-"hearts" of *Notonecta* are obtained graphically by plotting *log frequency* (= log [100/seconds for 10 beats]) against *reciprocal of absolute temperature.*

Preparations Nos. 29 (circles) and 30 (crosses), (the latter having the rates multiplied by 2.0 for comparison) give μ = 8,800. It may be noted that the latitude of variation, with the large scale-units employed, corresponds to an extreme difference, at the left end of the graph, of only 1.4 seconds in a mean of 23.4 seconds for ten pulsations.

In this way we have examined a large number of preparations, of which about 30 were studied in detail. The total number of observations was above 3,000. Since we desired especially to know the kinds of critical increments which might appear during the onset of death, it was necessary to avoid so far as possible the production of irreversible effects by exposures to very low or to very high temperatures. It was also necessary to work rapidly in order to make sure of time in

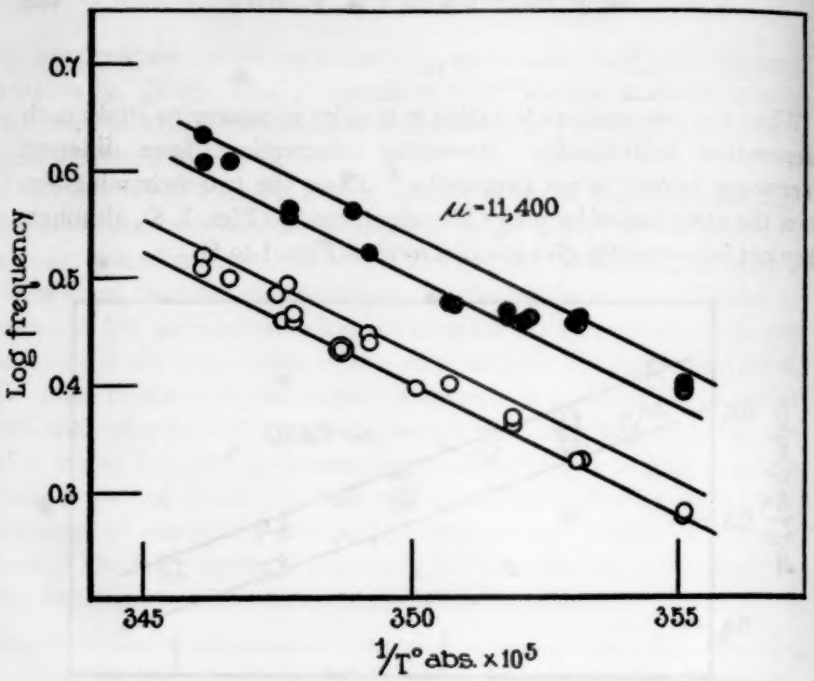

FIG. 2. Legs Nos. 27 (open circles) and 28, from the same individual, give μ = 11,400.

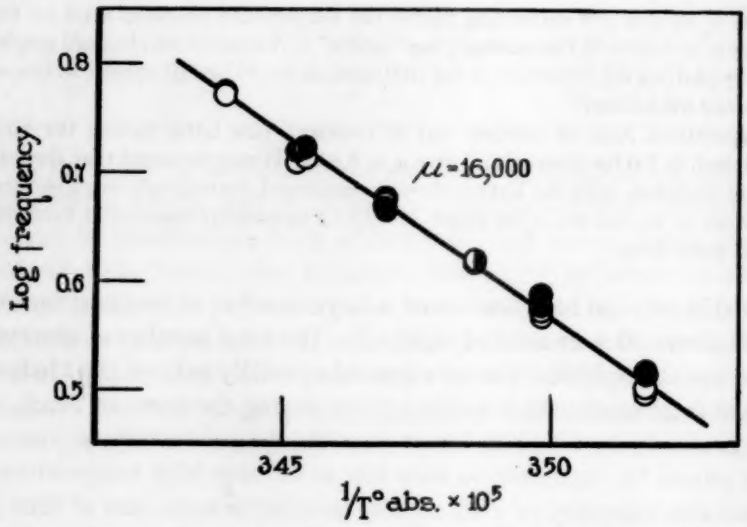

FIG. 3. Legs Nos. 19 and 20 give sensibly identical results; μ = 16,000.

which to travel in reverse order the excursions up or down the temperature range before intrinsic change of temperature characteristic should supervene. The absence of great natural variation in rhythm, coupled with the non-interference of spontaneous activities of the leg musculature, greatly facilitated such observations. The agreements in values of μ obtained show that confusion from these sources was successfully avoided.

The lower critical temperature for continued pulsation was 5°; in the isolated legs 20° was found a very definite upper limit for regu-

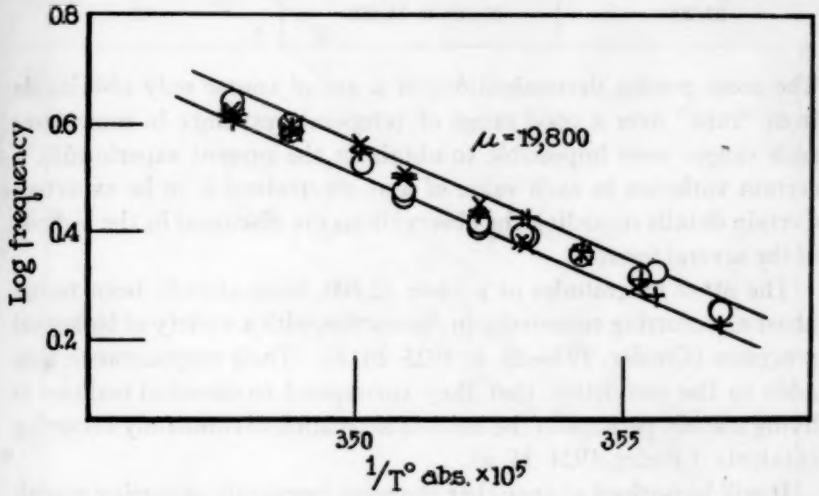

FIG. 4. Three preparations, Nos. 50, 51, and 52, give μ = 19,800. (The rates for No. 51 have been multiplied by 1.102, and those for No. 52 by 1.026.) These were from different individuals.

lar rhythm. In a number of preparations pulsations ceased at 9.5° ± 1.0°. The intermediate temperatures at which "breaks" appeared, indicating change of increment or of frequency, varied from 10.8° to 17.2°; the reason for this variation, as subsequently discussed, is found in the progressive exhaustion of the pulsating system, with induction of physicochemical changes independently of the thermal ones.

V.

The results of these observations are sufficiently illustrated by the examples given in Figs. 1 to 11. Including those instances in which

"breaks" occurred, as exemplified in Figs. 6, 8, and 11, the following is a summary of the critical increments found to describe the change in frequency of contraction as determined by temperature.

Mean μ	Extreme range	Number of instances
8,190	7,900 to 9,150	14
11,350	11,290 to 11,500	4
16,200	15,100 to 17,000	16
19,800	19,600 to 20,000	4
24,530	23,000 to 25,000	5
32,200	30,000 to 34,000	12

The most precise determinations of μ are of course only obtainable from "runs" over a good range of temperatures; since in some cases such ranges were impossible to obtain in the present experiments, a certain variation in each value of μ as ascertained is to be expected. Certain details regarding the observations are discussed in the legends of the several figures.

The other magnitudes of μ, save 32,200, have already been recognized as occurring repeatedly in connection with a variety of biological processes (Crozier, 1924–25, *b;* 1925–26, *b*). Their reappearance here adds to the conviction that they correspond to chemical realities in living matter, perhaps to the heats of activation of commonly occurring catalysts (Crozier, 1924–25, *a*).

It will be noticed at once that the most frequently occurring magnitudes are, approximately, 8,000, 16,000, and 32,000 calories. Bliss (1925–26) has described a case in which temperature characteristics of these general magnitudes apply respectively to different parts of the total temperature range as affecting the time required for a particular developmental phase in *Drosophila*. This kind of progression tempts speculation. The additive nature of heats of activation is recalled. But we believe that for the present the fact may most safely be recorded devoid of speculative fringes. This is especially so because if the individual records are examined it is found that with any one preparation there is no orderly association or succession of values such as 8,000; 16,000; 32,000 in different parts of the temperature range, or at different times. The increment 16,000, for example,

may be associated with 32,000, or with 8,000; or 8,000 with 32,000. This speaks for a certain random association of the processes manifested by the appearance of the respective increments; with this restriction, however, the lower of two increments always pertains to the higher temperature range.

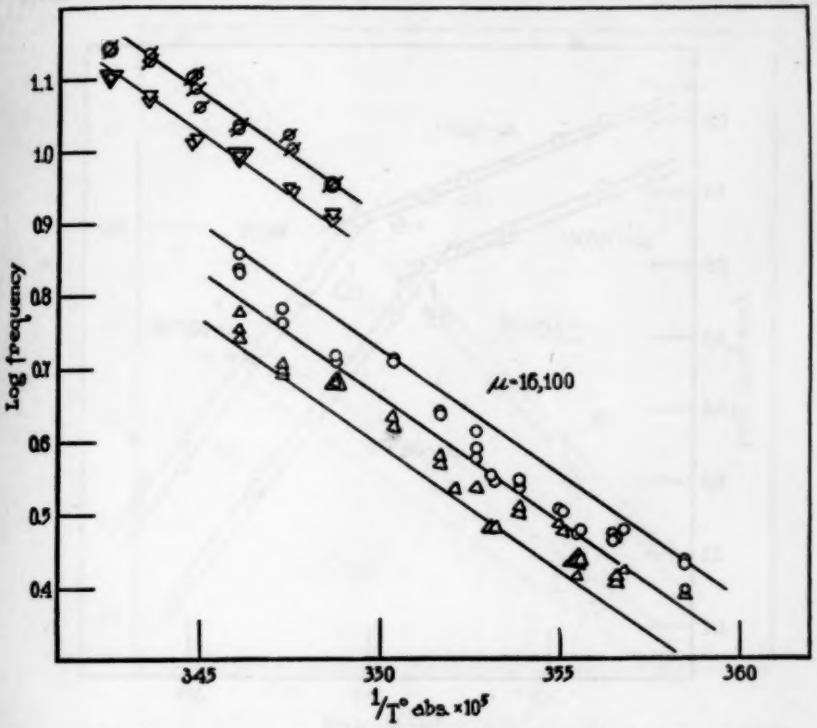

FIG. 5. Leg-"hearts" Nos. 31 and 32, from the same individual, give $\mu = 16{,}100$. The rate changed markedly after 24 hours, without noticeable change of μ, and by about the same amount in each leg.

In an isolated and irreversibly deteriorating system such as is presented by the isolated leg-"heart" it is to be assumed that changes in μ should also be evident as a function of time.

By "a change which is a function of time" we mean to imply that the processes of exhaustion and death should influence the occurrence of "breaks" in the relation between pulsation-frequency and tempera-

ture. The simplest illustration of such an effect is given by cases such as that shown in Figs. 5 and 8. It is obvious that the differential effects of exhaustion upon each of several processes such as might possibly control the frequency of pulsation may result in these elements of the nexus governing pulsation being successively revealed as determining the relationship to temperature. In numerous cases

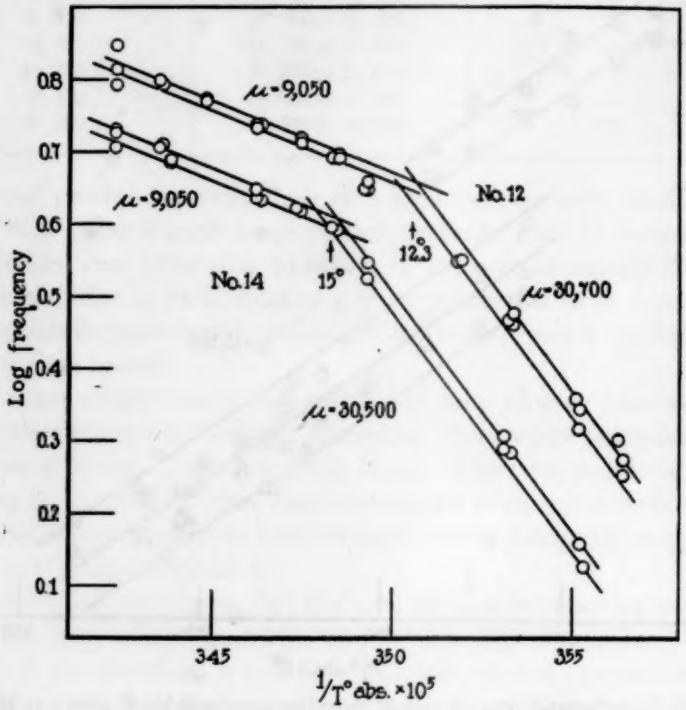

FIG. 6. Two preparations from different individuals, show transitions from $\mu = 9{,}050$ to $\mu = 30{,}700$ at lower temperatures.

where this sort of result is evident, reversibly, on passing from one zone of temperatures to another (Crozier, 1924–25, *b;* 1925–26, *b*) it has been assumed that it may be due to the fact that a catenary chain of reactions underlies the determination of frequency of pulsation (Crozier, 1924–25, *b*); the fact that the temperatures at which these shifts occur are not distributed at random (Crozier, 1925–26, *a*)

makes it necessary to suppose that physical changes, essentially of a grossly discontinuous character, are also implicated. In dealing with the *Notonecta* leg-"hearts" it was expected that the degradation of the excised system would have similar effects, and that in consequence the occurrence of an intermediate critical temperature should be

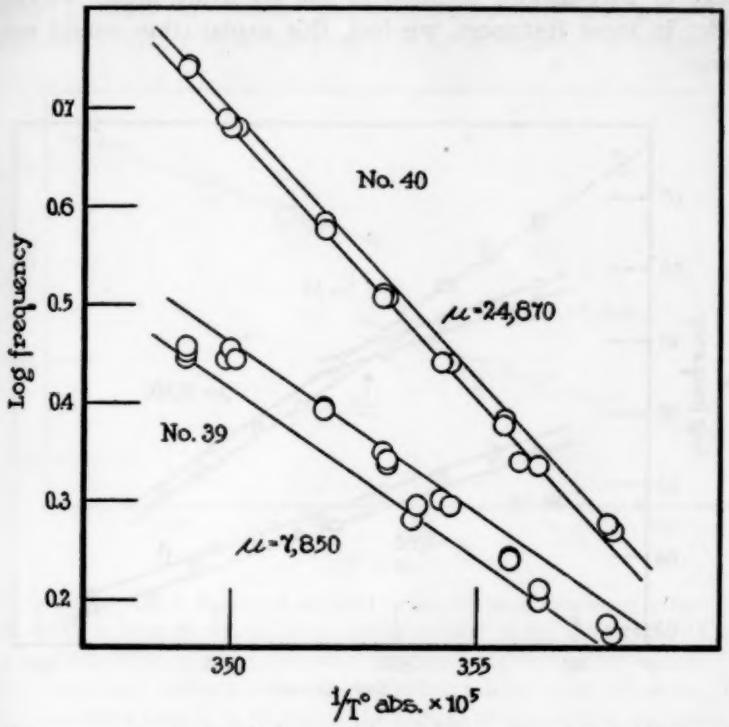

FIG. 7. Two leg-"hearts" (Nos. 39 and 40) from one individual give, respectively, $\mu = 7{,}900$ and $\mu = 24{,}900$. (The rates for one (No. 39) have been divided by a factor, 1.4, to permit unconfused plotting.)

blurred. This is the fact, but there is ample evidence, nevertheless, that in the neighborhood of 15° (10.8° to 17.2°) abrupt changes most frequently occur in the relationship between rate and temperature.

A further sort of complication in these curves is due to what we have characterized in other instances (Crozier and Stier, 1925–26, *a*) as a change of frequency or velocity without change of increment, or, what

is probably connected therewith in an intimate way (Crozier and Stier, 1925–26, *b*), a change in the latitude of variation at constant temperature without change of increment. This type of effect occurs in certain *Notonecta* preparations (Figs. 9, 10, 11) and might easily confuse interpretation. To what extent these shifts may be due to change of pace-making location in the pulsating organ we cannot decide; in most instances, we feel, this explanation would not be correct.

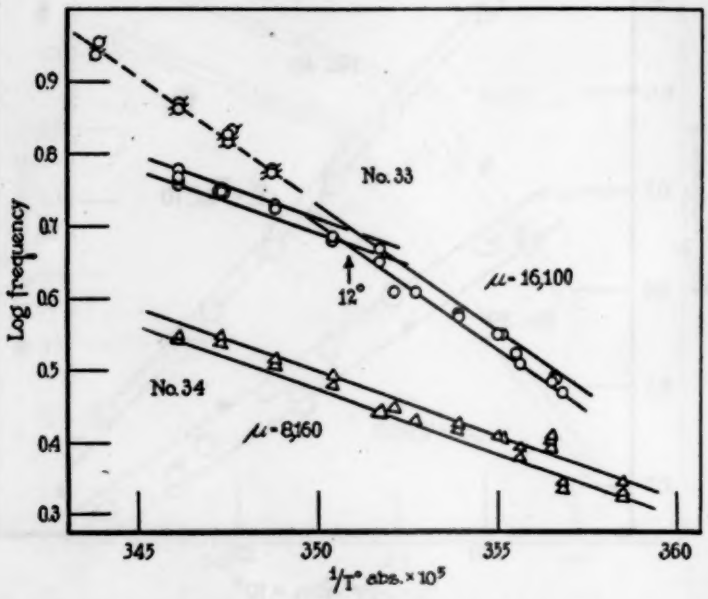

FIG. 8. Two preparations from one individual give, in one case μ = 8,200, in the other μ = 8,200 above 12°, 16,100 below 12°; on reversing the course of the temperature changes (dashed line), next day, the increment 16,100 is now found to hold in this case above 12°. This type of change is one of those presumed to depend upon the irreversible progress of exhaustion in the isolated legs.

VI.

The results of these experiments have been considered in terms of regularities in the nature of the influence of temperature on the frequency of pulsation in the leg-"hearts" and in a wide variety of other

objects. The magnitudes of the Arrhenius constant are not distributed at random. For this the explanation has been proposed that constants E or μ serve to characterize catalytic reactions governing the frequency of pulsation. It is possible to suggest a number of reasons why this sort of regularity "ought" not to be found. One method of dealing with the direct observations is to invent empirical formulæ for their expression. One such has recently been proposed by Bělehrádek (1926, *a*, *b*) in the form $y = a/x^b$, where x = temperature

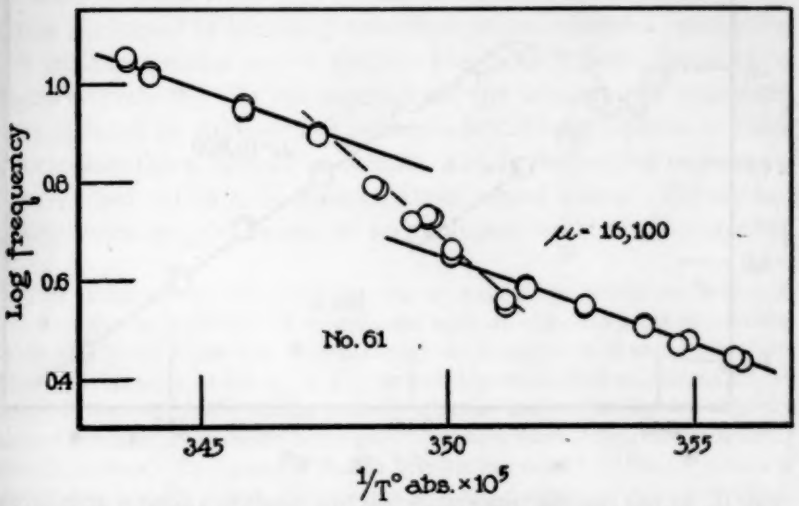

FIG. 9. A "break" in the curve of temperature relations was found in four cases, one of which is here shown, which is similar to that earlier described by Crozier and Stier (1924–25,*b*) in connection with the breathing rhythm of Anurans. The course of the observations was exactly reversed on raising the temperature. (The slope of the fitted lines (μ = 16,100) is the average of those fitting the upper and the lower segments.)

(*Centigrade*) and y = time necessary for a given phenomenon, and a and b are constants. This is obviously the well known empirical formula of Esson (Harcourt and Esson, 1895; Harcourt, 1912) for chemical reactions, with the substitution of the Centigrade temperature for T° *abs.;* it is difficult to conceive cogent reason, theoretically, for the proposed change, which simply requires making one of the constants larger without at all improving the fit. In addition to the

fact that this particular formula is of a type which can be made to describe *almost* any sort of curve not possessing too abrupt discontinuities, and that at best it has simply the status of an interpolation formula, there is the insuperable objection that it does *not* fit the facts. To illustrate this we may choose examples in which a large number of observations are available (Fig. 12). The statement (Bělehrádek, 1926, *a*) that a single simple curve can describe instances of the sort

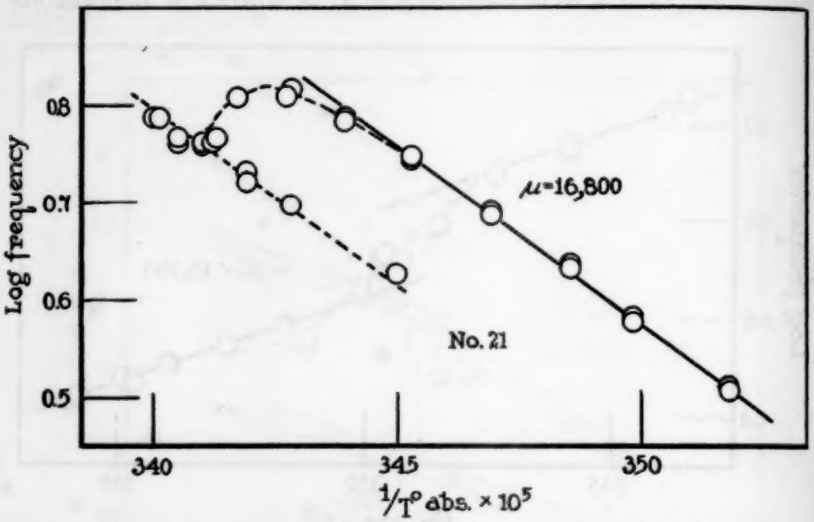

FIG. 10. In this case the temperature was first raised to a point a little above 20°; there was thus induced a permanent increase in pulsation rate, without apparent change of increment. Precisely this effect was obtained in other instances. The temperature was changed at intervals of about 15 minutes, between readings, and in the region of changing rate the points consequently fall on a curve. It is to be understood that the whole extent of vertical shift would have occurred at constant temperature.

shown in Fig. 6 is obviously futile (*cf.* Brown, 1926–27). The attempt to employ his formula has moreover led Bělehrádek (1926, *b*) into the assertion that his constant *b* reflects the primary importance of viscosity in connection with the velocities of vital processes, and that it changes systematically with the age of the organism. The notion that the temperature coefficient of a given activity decreases or increases regularly with the age of the organism is simply untrue, as our

own experience with a considerable number of cases enables us to state quite definitely. We make no apology for choosing to rely upon the one type of equation which actually describes the data and which seems to stand a good chance of physical interpretation; and until some other comprehensive explanation is provided for the regularities which its use discloses we are not impressed by objections grounded, largely, on the argument that protoplasm is "too complex."[3] The efficient answer to such very general objections is, that the complexity, or rather haziness, frequently resides in the mind of the observer, and can be dissipated by increased refinement of experimental procedures.

A more interesting sort of obstacle has recently been discussed by Murray (1925–26). In his experiments the temperature characteristics deduced for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and, in the number of preparations studied, failed to be grouped about modal values. We are not familiar with the performance of such cultures, but it can be suggested

[3] It is perhaps of interest, since the present material enables several points of some moment to be illustrated, to comment upon an objection sometimes voiced to the practice of fitting two or even three lines to segments of such data as are plotted, for example, in Fig. 6. It may be said that the implied transitions are too sharp. If one were really dealing with the effect of temperature upon a catenary series of reactions there should be a region of curvature connecting the two sensibly rectilinear zones. The answer is that in suitable instances just this indication of curvature is actually found. Most series of measurements exhibit a latitude of variation which makes it difficult to decide the precise nature of the union between the two straight lines. A number of series in the present experiments (*e.g.*, Fig. 6) do however show this sort of "rounding off" when the latitude of variation is quite small. Objections to considering the whole sweep of such series as a single curve have been mentioned in a preceding paper (Crozier and Stier, 1926–27, *a*). Sharp transitions of the sort implied in these broken graphs are of course well known in physics; we may refer to transition points, and to curves of magnetic susceptibility. Moreover, as illustrated in a recent paper from this laboratory (Brown, 1926–27), the data so fitted cannot be described by a single smooth curve when plotted directly as *velocities* against *temperature*. Again, as has been insisted previously (Crozier, 1925–26, *a*), there is indication of "physical" alterations occurring at just those temperatures most frequently found to be transition points on the thermal scale. The reason for the occurrence of just these critical temperatures must be sought in the properties of protoplasm as a physicochemical system.

that in such a preparation, essentially an unorganized mass structurally though exhibiting regularity as to pulsation, there must exist a number of possible pace makers. It is to be expected that the net result of their fluctuating dominance might obscure the preponderating in-

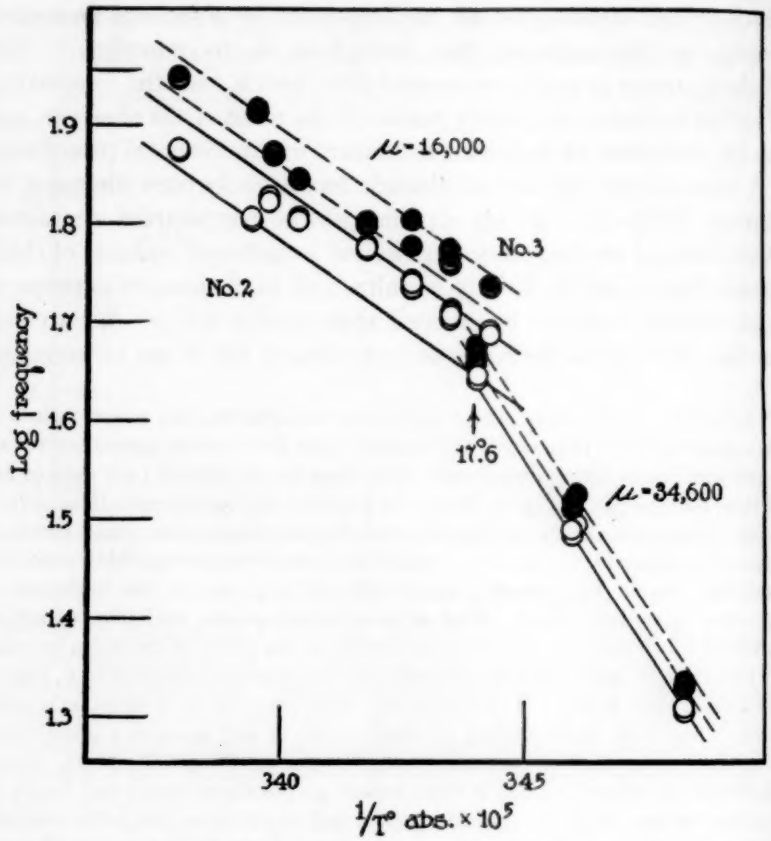

FIG. 11. Occasionally, instances were found in which an abrupt change of increment was associated with an abrupt increase of rate.

fluence of any one, since it is fair to assume that their several inner metabolic states might be differently adjusted. As we have pointed out in detail in a following paper, this interpretation permits certain deductions which the figures in Murray's paper seem to us to justify.

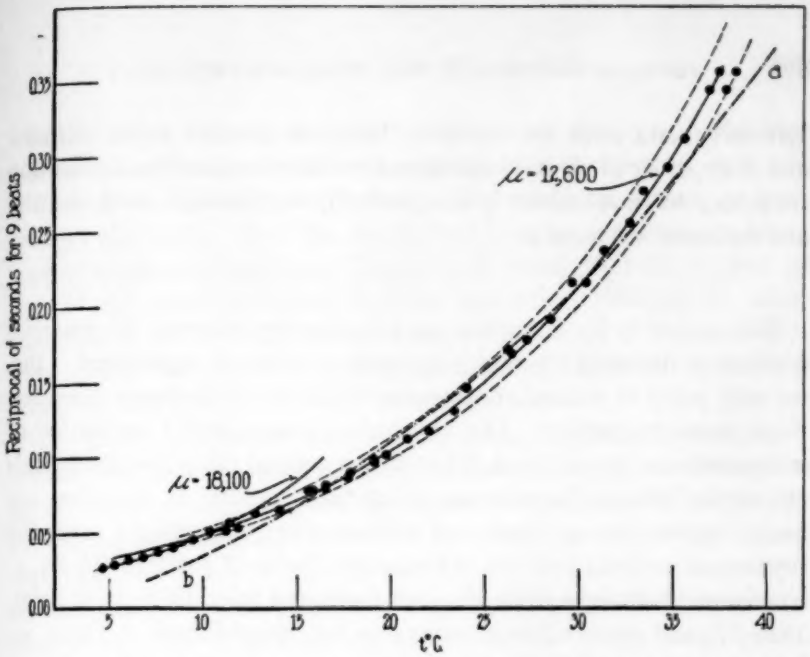

FIG. 12. Data from one experiment (in Fries, 1926–27) on the frequency of cardiac contractions in *Blatta* are fitted by the Arrhenius formula with $\mu = 12{,}600$ between 10°C. and 38°C. In the original presentation (Fries, 1926–27, Fig. 2) these data appear plotted as *log frequency vs.* $1/T°$ *abs.*, where the nature of the "break" at 10° is more clearly apparent; below 10°, $\mu = 18{,}100$. The dashed lines are transposed from the lines defining the extreme latitude of variation in the log plot (Fries, 1926–27, Fig. 2). The superiority of the logarithmic representation (*vs.* $1/T°$ abs.) consists not only in the clearer appearance of conditions on either side of a critical temperature (*e.g.*, 10° in this case), but also in the fact that the departures from the line of best fit are confined within a band the upper and lower edges of which are parallel to the central line (*cf.* Crozier and Federighi, 1925). The latter fact means that it is necessary to deal, in fitting the Arrhenius equation to such data, not with the absolute departures of the observed average rates or frequencies, but with the relative or proportionate divergences; this is of great importance if it be suggested that curves such as that in the present figure should be fitted by the method of least squares (*cf.*, also, Crozier and Federighi, 1924–25, *b*, 1925).

To curves obtained by the method just described there has been added (*a*, *b*, — · — · — · —) the result of an attempt to fit the observations by means of the formula proposed by Bělehrádek (1926, *a*). This formula, $time = A/(t°\text{C.})^B$, (or $rate = (t°)^B / A$,) tested by appropriate plotting of the observations as *log rate vs. log* ($t°C.$); in such a plot the best fitting straight line was adjusted, and has then been transferred to the present figure. It is obvious that the fit is anything but significant; nor can the adjustment of the ends of the curve be made such as to improve the fit; from the nature of Bělehrádek's formula, correction of the lower end of the curve, for example, merely increases the deviation at the upper end.

Extensive data upon the myogenic heart of *Limulus* larvæ (Crozier and Stier, 1926–27, *b*) have convinced us that the relation of pulsation frequency to temperature is of a perfectly regular sort, with definite and recurrent values of μ.

VII.

This paper is by intention an introductory account of material which it is designed to employ for certain types of experiment. But we may point to several conclusions which the preliminary examinations seem to justify. The rhythmic neuromuscular activities of arthropods, so far as tested, fall into two general categories as regards the critical thermal increments which they reveal. In one class are found movements of heart and of locomotor appendages and the rhythms of stridulation and of luminous display (Crozier, 1924–25, *a;* Crozier and Federighi, 1924–25, *a, b*; Crozier and Stier, 1925–26, *a;* Fries, 1926–27; and some other instances as yet unpublished). These we have reason to regard as controlled by nerve centers, and they show a high degree of consistency in providing μ = 12,200 to 12,500. Occasionally this value is associated with a higher one over the lower portion of the temperature range, μ = 18,200 or 23,500. On the other hand, movements of respiration (Crozier and Stier, 1924–25, *a;* and other data unpublished), presumably also determined by nerve center activity, but demonstrably of a different kind, consistently yield other increments,—those, namely, associated with cell respiration itself (Crozier, 1924–25, *b*).

The isolated leg of *Notonecta* with its "heart" is remote from central nervous control of the type pretty certainly involved in the movements of the dorsal vessel (*cf.* Alexandrowicz, 1926). We are not yet able to say just what the thermal relations are in the intact animal, but in the isolated leg-"heart" the increment 12,300 $\pm$ does not appear among the several values encountered. On the other hand, the values actually found (Table I) are those characteristic of oxygen utilization, heat production, and CO_2 production. We do not conclude from this, of course, that the leg-"hearts" are therefore concerned with respiration, but merely that the metabolic activities underlying and controlling the rates of pulsation are certainly different from that determining the increment 12,300.

It is notable that in material of this sort we should rather expect serious disturbance of rectilinear relationships between *log rate* and $1/T$, such that plainly curvilinear graphs would be gotten, or progressive shifts of μ. But the fact is that here, as in the case of grasshopper respiratory motions (Crozier and Stier, 1924–25, *a*) and in that of the heart of *Limax* (Crozier and Stier, 1925–26, *b*), when change of μ occurs it takes place abruptly and by a definite amount. This speaks strongly for the individualized character of the several processes which may control the rate of pulsation.

The presence of increments often associated with respiratory phenomena led us to attempt to alter the increment by controlling the oxygen supply. We were unable to obtain consistent differences by comparing hearts in legs immersed in water through which O_2 constantly bubbled, legs swathed in vaseline, or legs sealed in tubes with a small volume of water. The differences found are for the greater part attributable rather to the metabolic condition of the whole insect. This we expected to find influenced by laboratory confinement. It is of interest, therefore, to note that the increments 32,000 ± were chiefly, although by no means exclusively, obtained from individuals not more than 2 weeks after collection. The increments 19,800 and 23,800 appeared only in the cases of legs from *Notonecta* kept for some 2 months or more in the laboratory.

VIII.

SUMMARY.

The frequencies of pulsation of the "accessory hearts" in the isolated swimming-legs of *Notonecta* were studied in relation to temperature, with the idea that in such organs central nervous control is impossible, and that in an isolated system irreversibly proceeding toward death it might be expected that further evidence would be found regarding the supposed specific significance of critical thermal increments. A number of values of μ are found, commonly 8,200; 16,200; or 32,200; less frequently 11,400; 19,800; and 24,500. These values are definitely contrasted with that (12,300) typical for heart beat frequencies in arthropods. They exhibit interrelationships of the sorts already found in other cases. There occur also sharp irreversible changes in

frequency of pulsation, which may or may not be accompanied by change of increment. The net result is held to be confirmatory of the interpretation of thermal relations proposed in earlier papers.

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TEMPERATURE AND FREQUENCY OF CARDIAC CONTRACTIONS IN EMBRYOS OF LIMULUS.

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I.

The heart of *Limulus* is classic as example of neurogenic cardiac rhythm (Carlson, 1905-06, 1909). The frequency of its pulsations may be controlled by influences affecting the heart ganglion alone. When the temperature of the ganglion alone is varied (Garrey, 1920–21, *a*, *b*, *c;* 1921–22) the frequency of contractions adheres to the Arrhenius formula, μ in the equation

$$Frequency = Ke^{-\frac{\mu}{RT}} + C$$

being = 12,200 (Crozier, 1924–25, *a*); in some preparations there occurs a "break" at 15°, with μ at lower temperatures = approximately 23,500.

In the embryo of *Limulus* there is an early developmental period in which the heart is visible, contracting rhythmically, while the cardiac nervous system is still unformed; during this interval the heart rhythm is "myogenic" (Carlson and Meek, 1908). We have determined the relationship between temperature and frequency of heart beat during this period of "myogenic" rhythm, in order to compare the temperature effect with that in the heart ganglion of the adult. There seem to be clearly defined differences.

There is no reason to suppose that a difference in μ obtained in this way necessarily corresponds to or is diagnostic of myogenic as contrasted with intrinsically neurogenic processes. In so far as values of μ appear to be specific, and thus to correspond to physical realities

which may be utilized for purposes of classification and analysis,[1] it must be held that particular magnitudes of μ may reappear in all sorts of situations and do not pertain individually to particular types of function. But at the same time it is apparent that the occurrence of different systems of temperature characteristics for the two cases, embryonic heart and adult, is fully consistent with the idea that the respective essential controlling processes are unlike.

Such a result is of course not unexpected. The metabolic state of embryonic cells must differ materially from that of relatively greater dynamic stability enjoyed by the protoplasm of fully differentiated tissues. From this standpoint the apparently "irregular" variation of μ for rhythmic contraction in cultures of explanted chick myocardium (Murray, 1925–26) might be understood without reference to obscure regulation by the organism as a whole in order to account for greater uniformity in results when organs of intact animals are observed, even without appeal to structural conditions. When whole organisms are used it is possible to obtain modifications of temperature characteristics (μ), and these modifications appear to be specific (Crozier and Stier, 1924–25, *a;* 1925–26, *b*). It is entirely possible that the regularity of μ for comparable activities is partly determined by structural conditions in normal organs, such as permit of active control by definite "pace makers." Certain effects which seem to necessitate this view are discussed in a later section. It is perhaps of interest for this interpretation that the frequency of pulsation in the hearts of intact embryos may show considerable differences in μ among similar individuals (*cf.* Crozier and Hubbs, 1924, and other cases), or in relation to age and other variables. The present observations show differences between individuals comparable to those experimentally induced in the breathing rhythm of the grasshopper (Crozier and Stier, 1924–25, *a*).

[1] Crozier, 1924–25, *a*, *b;* 1925–26, *b*. Crozier and Stier, 1924–25, *a;* 1925–26, *a*, *b*. Fries, 1926–27. It may be noted that change of μ coincident with the institution of neurogenic control would not necessarily prove diagnostic either, for we should require study of comparable developmental stages in the absence of nervous elements.

II.

The heart of *Limulus* embryos within the egg envelopes becomes visible as a pulsating organ at the stage labelled *H* in accounts of the differentiation of the embryo (Kingsley, 1893; Kishinouye,1893). For a period of about 6 days, or until Stage *K* (before the appearance of the telson), at laboratory temperature (Carlson and Meek, 1908), the activity of the heart continues to be visible, in the absence of nervous control.

The heart is not at any time particularly easy to see. This difficulty, together with expected variation in the effect of temperature, led us to practice special precautions in obtaining a large number of observations. The frequency of the heart beat is made visible by horizontal light of fair intensity, under which the cardiac tube appears as a delicate white ghost against the yellowish background of the substance of the embryo. In order to maintain the animal in a position suitable for observation, the egg membrane was punctured and a segment of it folded outward. This segment was fastened by white vaseline to a small glass block. The collapsed membrane holds the embryo in a relatively fixed position. Such preparations live in an apparently normal way for many days and continue to develop.

A number of glass blocks, carrying labelled embryos, are placed in a thin walled crystallizing dish with sea water. Into this projects a microscope with paraffined objective used as an immersion lens. Dish and microscope are securely fastened to an iron frame supported on the rim of a large water thermostat. The vessel containing the embryos is submerged so that its water level is below that of the water in the thermostat. The temperature is read on a thermometer with enclosed stem, calibrated, and graduated to 0.05°.

In all such experiments difficulty is met in maintaining temperatures below that of the room. The latitude of fluctuation in frequency of contractions, at constant temperature, necessitates a number of observations at uniform temperatures on each embryo. Yet it is required to change the temperature by a known small amount at intervals of about an hour.

To do this we constructed a thermostat from which heat could be abstracted by a SO_2 compression circuit. The coil and brine tank of a refrigerating unit were replaced by a considerable length of half-inch Cu tubing, coiled so as to form a helical shell within the wall of a 10 gallon glass tank. The motor operating the compressor was started through a relay actuated by a large mercury thermoregulator.[2] "Sticking" and sparking were obviated by using a Ni-steel needle to make contact with the fluctuating Hg surface, and by having the relay of high

[2] For aid in this construction we are greatly indebted to Mr. H. V. Rivinius, refrigeration engineer of the Metropolitan Ice Co.

resistance (5000 ohms).[3] With adequate stirring, and felt insulation, this device enabled us to maintain for as long as desired any required temperature between 0° and that of the room. When an electric heater, constant or relay-controlled, is added to this arrangement, temperatures above that of the room are similarly obtainable, and the slight lag in temperature adjustment is still further reduced. The constancy of temperature within the body of the thermostat is then ±0.001°. In the vessel carrying the objects to be studied the constancy is well within 0.01°, and this is improved by a cover.

The temperature is changed quickly by adding hot or cold water from a reservoir, excess in the thermostat being removed by a constant level device. The thermoregulator is readjusted by sucking Hg out of the regulator bulb, or by forcing more Hg into it from an accessory bulb. Finer adjustment is made by the screw-mounted contact needle. This operation required but a minute or so.

The thermostat is mounted upon a box, and a window is left in the insulation of the bottom. Through this window a beam of light is projected vertically upward, and may assist observation through the microscope or be employed in other ways.

The microscope, of ordinary type or a binocular with "Planktonsucher" objectives, is so mounted as to be movable over the observation chamber; but in the present experiments it was found that the glass blocks bearing embryos could easily be manipulated with a needle and in turn brought into position for the readings. These movements were found to be without influence upon the frequency of the heart beat, but some minutes were allowed to elapse before readings were taken. Lateral illumination was supplied by a small submerged electric lamp. It was easily shown that the light was without effect upon the frequency of contractions, but the general illumination was kept reduced as an aid to seeing the heart, thermometers, and thermoregulator being viewed by means of small accessory lamps.

III.

When the frequency of the heart beat or of breathing movements is to be timed with precision in an intact animal it is necessary to avoid carefully the effects of concurrent movements of the body or appendages.[4] In some instances it seems as if the execution of such movements is the cause of accelerations or retardations in the rhythm under observation; in others, it appears more probable that both disturbances have a common and simultaneous origin in the central nervous system. If it is desired to study intrinsic fluctuations of frequencies and to obtain temperature characteristics as precisely

[3] Regulation may also be conveniently made by means of a system such as that described by Beaver and Beaver (1923).

[4] *Cf.* Crozier and Stier, 1924–25, *a*.

as possible, such deviations must be taken account of. In the *Limulus* embryo, as in other embryos, there are evident from time to time "spontaneous" movements of body and legs. But it happens that in *Limulus* these movements, like the photokinetic movements of the legs which appear when the light intensity is suddenly changed, seem to have no influence whatever upon the sequence of the heart beats.

The frequency and the amplitude of the cardiac movements nevertheless go through a rather definite cycle, at constant temperature. This was ascertained by repeated observations on single individuals; since the data add nothing new to the theory of the case, they are not given here. The latitude of variation is slightly greater than 10 per cent of the mean frequency, and, as in some similar cases earlier described, is constant over the workable temperature range but varies from one individual to another. The latitude of variation seems quite unrelated, in general, to the relative rate of the process considered, and since it varies within pretty narrow limits for a variety of activities (5 to 10 per cent of the mean), it must be regarded as chiefly determined through some property of protoplasmic organization rather than by the specific process whose temperature characteristic is being measured. In a small number of known cases (Crozier and Stier, 1926–27, *a*) the latitude changes when μ differs on either side of a critical temperature, and in such cases a specific association must be assumed.

The slow developmental pace of the *Limulus* larva, together with the great resistance to asphyxiation (*cf.* Kingsley, 1893; Newman, 1906; Redfield and Hurd, 1925) contribute to its suitability for our purpose. The majority of the individuals used were kept at 20°, when not subjected to temperature changes experimentally. No differences were seen in other embryos maintained at 4.5° for several weeks. It was possible in this way to have embryos of various stages of development available at one time. Reversing the course of the temperature changes gave observations in good agreement. Within certain limits duplicate "runs" on successive days also agreed well; but as a rule the frequency of heart beat changed after 1 or more days, although—so far as ascertained—without change of temperature characteristic. The total number of observations was 3400.

IV.

Sixteen series of readings, on fourteen different individuals, were well controlled by repeated check observations at the same tempera-

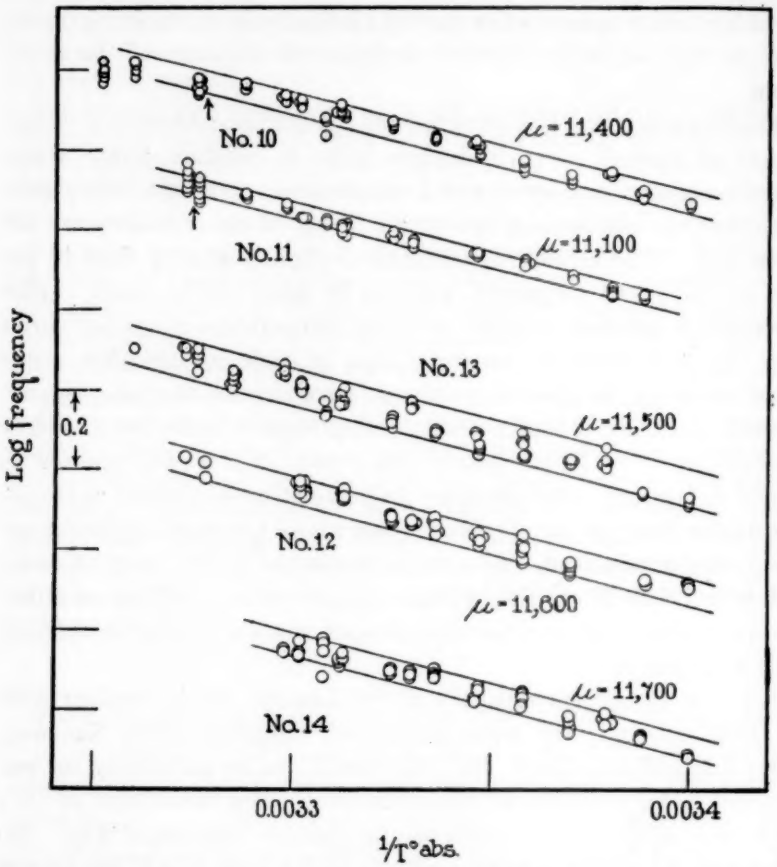

FIG. 1. Observations on the frequency of heart beat in five embryos of *Limulus*, at temperatures between 20° and 35°±, for which μ = 11,400. (The frequencies at constant temperature are very nearly the same; the frequency is taken as 100 ÷ seconds for ten beats.)

tures. Of these individuals seven provide increments ranging from 11,000 to 12,280 (the latter value is probably too high); for these the average μ = 11,520 ± 100. Three series gave μ = 16,430 ± 200.

Two gave μ = 20,000 ± 100, and two μ 25,500 ± 300. When data from any one animal are considered over a range of temperatures the lower value of μ pertains to the higher temperature interval (20°–30° +), but one embryo gave μ = 11,000 over the range 10°–20°, and μ = 16,400 occurs both in the range 10°–20° and in 20°–30° (with different embryos).

Above 30°–34° the rate of increasing frequency of heart beat with elevation of temperature is very slight; most embryos of this

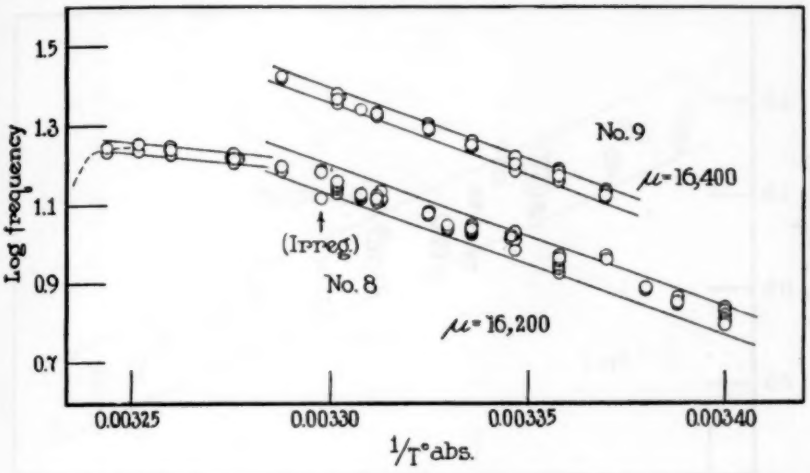

FIG. 2. Data from two *Limulus* embryos for which μ = 16,300 (20°± to 30°±); the rates for No. 9 have been multiplied by 1.59. The latitude of variation, as seen also in Fig. 1, varies with the individual. Above 30°± the frequency changes very little with increasing temperature; the curve, within the region shown (No. 8) is perfectly reversible. This phenomenon has already been referred to as apparent in other cases (*cf.* Crozier, 1925–26, *a*).

age show slight but easily detected decreases in frequency of heart contraction, which are only very slowly reversible on return to temperatures below 30°. Other individuals do not exhibit this hysteresis, but above 30°± the thermal increment is very small (μ = 5,000 ±) between 30° and 40.5°. This effect resembles that already noted in some other instances (Crozier, 1925–26, *b*), and is suggestive of the control of heart beat frequency by some purely physical condition, such as fluidity of substance or the saturation of some reactive sur-

face which gives a mechanical limit to the maximum frequency of pulsation. In one case which is illustrated (Animal 2, Fig. 5) no hysteresis was apparent on return to lower temperatures.

The temperatures for cessation of regular cardiac rhythm were 9° and 45°; above 40.5° the frequency of contraction decreases; at 44.4° the heart beat was still regular, but at 45.4° only an occasional beat was apparent. For the adult *Limulus* (Carlson, 1906) the thermal limits for contraction of the heart muscle are given as 0°± and 32°,

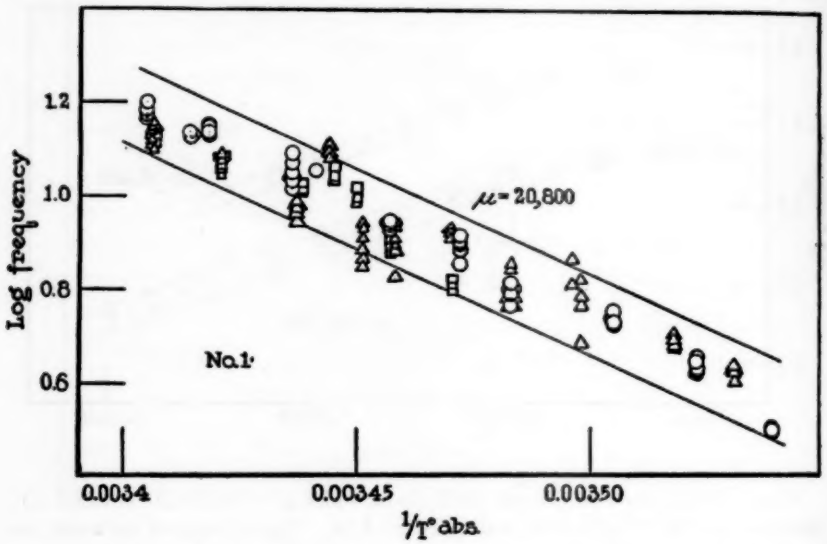

FIG. 3. One individual gave data (three "runs" of observations) yielding μ = 20,800 for frequency of cardiac rhythm, below 20°.

with "heat rigor" appearing at 47°; for the cardiac ganglion and nerves, the limits of activity are 0°– and 42°–43°. At the lower temperatures, beating was observed to become regular at 8.7° to 9.2°; below 8.7°, down to 5.9°, an occasional isolated beat was seen; on warming up to 8.8° to 9.0°, regular contractions were uniformly observed. It is worth noting that these thermal limits, determined from observations upon a large number of embryos, were found to be the same in embryos kept for 15 days at 4.0° as in those maintained at room temperature or used in warming or cooling experi-

ments. This agrees with the essence of Mayer's (1914) findings upon the adult *Limulus* and points to the conclusion that in this instance the thermal effects depend upon the composition of the animal, rather than upon thermal adaptation.

Between the extremes of temperature which limit the exhibition of regular rhythm (9°, 40.5°) the following are found to be critical temperatures, in the sense (Crozier, 1925–26, *a*) that abrupt change or irregularity may there appear in the curve relating frequency to

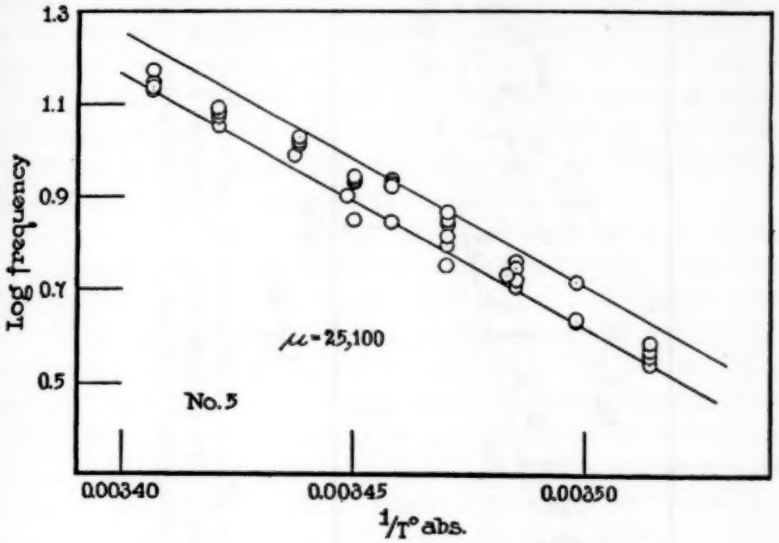

FIG. 4. *Limulus* Embryo 5 gave μ = 25,100, below 20°.

temperature: 20°, 27°, 30° to 34.5°. We purposely avoided the possible influence of the "break" at 20°± by largely working below or above this temperature, with different embryos. One individual (No. 2; Fig. 5) was found by repeated runs of observations to exhibit a sharp "break" in the curve of heart beat frequency at 20°–21° of such a character that a change in frequency accompanied a change of temperature characteristic. This is the first instance of the kind which we have been able to study carefully. The probable existence of such cases has earlier been mentioned (Crozier, 1925–26, *b*); they are of particular interest for the theory of critical temperatures.

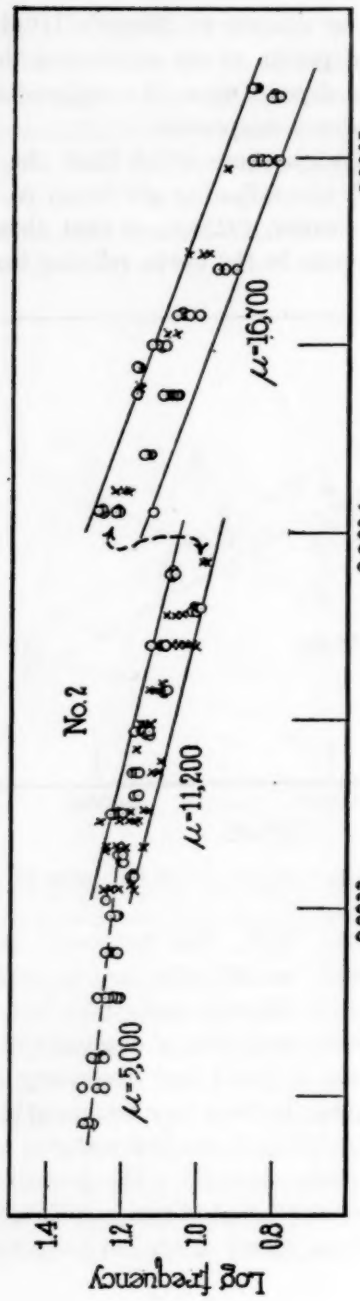

FIG. 5. Embryo 1 gave μ = 16,700 below 20°, μ = 11,200 between 20° and 30°; above 30° the curve flattens out (as with No. 8, Fig. 2, and in other instances not plotted). The particular interest of this graph is in the type of "break" found at 20° (see text). Two series of observations are distinguished by symbols; in the second series (crosses) the latitude of variation is less, below 20°, than in the first series.

V.

The temperature characteristics for frequency of cardiac rhythm in *Limulus* embryos thus appear as definite and recurrent quantities. The fact that two individuals apparently similar may yield quite different magnitudes of μ simply means, we take it, that the two pace maker cells or cell groups in these hearts have slightly different metabolic adjustments, or that the pace maker groups are different. If this were correct we might reasonably expect to alter μ experimentally in a more or less predictable way. This we did not attempt in the present observations although it has been accomplished in other instances (Crozier and Stier, 1925–26, *b*). It would also be predicted that the various values of μ obtained should show certain interrelationships. Thus the common association of the values 11,300 and 16,200 in respiratory and other processes (Crozier, 1924–25, *b*) finds rational application in the present case.

For the heart of adult *Limulus* the characteristic μ, so far as can be ascertained, is about 12,200; in several individuals (data from Garrey, 1920–21, *a*, *b*; *cf.* Crozier, 1924–25, *a*) μ = 23,500 below 15°. With one exception, which is not intrinsically of great weight, this value of μ does not appear in connection with the embryonic heart. The data upon adult heart rates came from experiments in which the temperature of the cardiac ganglion alone was varied, and the μ obtained agrees quantitatively with that for a number of other instances among arthropods in which central nervous control may be assumed (Crozier, 1924–25, *a*; Crozier and Stier, 1925–26, *a;* Fries, 1926–27). The increments apparent in the observations on the embryonic hearts, however, are of frequent occurrence in data on the heart rhythms of molluscs and vertebrates (*cf.* Crozier 1925–26, *b*). This sort of result points definitely to different chemical control of heart pulsation in embryo and in adult *Limulus*, and to the relative diversity of the pace-making control in the developing embryos. (If adult *Limulus* were to be used, the possibility of myogenic effects might have to be reckoned with, in addition to the neurogenic automatism, if the temperature of the whole organism were varied; *cf.* Hoshino, 1925.)

The possibility of diverse pace-making processes in the hearts of different individual embryos, and thus in different but functionally

analogous cells, is important for the understanding of thermal effects in isolated heart preparations and in cultures of developing myocardium. To this there must be added the recognition of sources of confusion which may result from the fluctuation of controlling circumstances within single cells, of which at least two kinds of disturbing effects can reasonably be suggested. The behavior of the "accessory hearts" of *Notonecta* is particularly significant in this connection (Crozier and Stier, 1926–27, *b*). At the moment we wish to deal particularly with the consequences of the occurrence in a single pulsating structural mass of a diversity of possible pace-making elements (*cf.*, for the chick heart, Cohn, 1925). Murray (1925–26) found that the apparent temperature characteristics for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and failed to be grouped about detectable modal values. In such preparations there must exist at any moment a number of possible pace makers. The net result of their fluctuating control would be expected to obscure or to blur the influence of any one, since it is fair to assume that their respective inner metabolic states might be differently adjusted. In heart cell cultures the controlling influence of the intrinsically faster beating component of compound masses has been demonstrated experimentally by Olivio (1926), and this appears indeed to be a general condition (*cf.* Mayer, 1911; Crozier, 1916).

The sort of situation, therefore, which we believe to exist in pulsating heart cell cultures is one in which a number of distinct "pace-making" cells or cell groups are present in each pulsating mass. The intrinsic frequency of initiation of rhythmic contraction is supposed to differ among these pace makers. If one pace maker definitely possesses a much faster rhythm than the others, its effect is uniformly apparent. But if two or more pace makers have nearly the same intrinsic frequencies, but are metabolically different, so that each exhibits a characteristic relation to temperature, their several influences upon the gross sequence of pulsations should interpenetrate; at one moment pace maker A, at another instant, before A starts again, pace maker B is in control. One consequence of this kind of effect may be tested immediately. The latitude of variation, expressed as a percentage of the mean pulsation-frequency at each

temperature, should not be constant if pace makers *A* and *B* have different temperature characteristics. This is precisely the situation disclosed in several of Murray's (1925–26) figures. Therefore temperature characteristics deduced from such data *en mass* must be regarded as without specific significance.

Three corollaries are at once deducible. (1) The situation here pictured and tested differs from that (Crozier, 1924–25, *a*) in which it is supposed that the slowest process of a catenary series of catalyzed transformations dictates the speed and temperature characteristic for the velocity of formation of the end result. In the present case the swiftest pace maker determines the maximum frequency of rhythmic contraction. Therefore we may expect to find cases in which the "mean" temperature characteristic is increased at higher temperatures. The realization of this possibility is suggested in several figures given by Murray (1925–26; Figs. 1, 2, 3). One effect of this would be to bring about instances in which the log latitude of variation increases with increasing temperature as well as others in which the change is in reverse direction. These differences appear in Murray's figures.

(2) Of greater interest is the corollary that if our assumed pace makers *A* and *B*, respectively generating the most frequent and the least frequent contractions among the group capable of acting as pace makers at all, should have the same temperature characteristic, then the logarithmic latitude of variation should be constant; and in such cases the μ deduced should correspond well with a value found in homologous instances. In the figures given by Murray (1925–26; Figs. 1, 2, 3) we find, for cases meeting this requirement, $\mu = 8{,}000$, $\mu = 11{,}000$, and $\mu = 16{,}100$; these compare well with values commonly recognized in heart rate measurements (Crozier, 1925–26, *b*).

(3) For cases in which the latitude of variation is inconstant, the slopes of the lines fitting the extreme variates on the semilog plots should be straight and should provide approximate μ values characteristic of the limiting pace makers. And we should expect these to compare favorably with temperature characteristics encountered elsewhere. We have treated in this way the plots given in Murray's Figs. 1, 2, 3. The result in one case is reproduced in Fig. 6. The

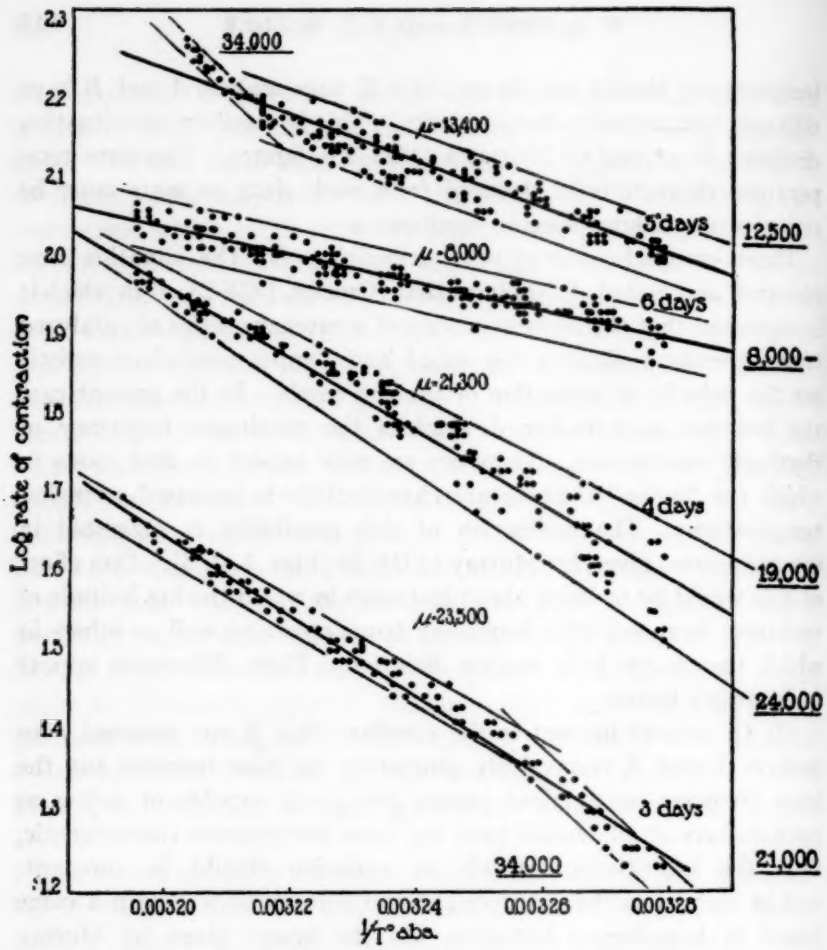

FIG. 6. Reproduced from Murray (1925–26, Fig. 2). The contraction rates of auricular fragments from embryos of the ages indicated. The central line in each plot is that originally given by Murray. To these lines there have been added marginal lines fitting the extreme variates. When attention is given to the latitude of variation it is obviously necessary to deduce values of the temperature characteristics somewhat different from those originally given. Values obtained from the marginal lines are indicated with underscoring. The nature of "breaks" in the uppermost and the lowermost graphs are fairly clear. When the log latitude of variation is constant, values of μ are gotten ("6 days," *e.g.*) which are already well known in other situations. When the latitude of variation changes continuously on the semilog plot the marginal rates are regarded (see text) as due to the operation of diverse pace makers. We consider that the recognition of these sources of confusion in curve fitting is sufficient to remove the force of the contention that temperature characteristics vary at random in this case.

characteristics obtained are noted in the figure, and the result may certainly be taken to agree with the expectation.

The effect of two such limiting pace makers could be imitated by combining the observations from two individuals (*Limulus*) in which μ for frequency of heart beat is different but the rates at given temperature approximately the same. It may be suggested that precisely this condition may appear if one were to measure the frequencies of contraction in the heart of an Ascidian, without reference to the places of origin of the individual beats; or, perhaps better, in a medusa deprived of all but several rhopalia.

These considerations do not completely account for the sources of complexity probably present when such an object as a heart cell culture is studied in this way. The indications already obtained, however, show why in these analyses we have continuously insisted (1) upon the errors which may be involved in the process of averaging rates or frequencies of vital processes in different individuals, or even in the same individual at different times, and (2) upon the ribbon form of significant plottings. There is to be added the further type of difficulty entering when a break occurs in the curve relating frequency or rate to temperature; should this sort of change be present in the activity of one pace maker, absent in others, the logarithmic latitude of variation must again change if the curve for this pace maker falls outside the limits set by the activities of other concurrently effective pace makers. Such a break, furthermore, may or may not be accompanied by an abrupt change of frequency; and changes of frequency may occur without change of temperature characteristic. These are not imaginary situations (*cf.* Crozier, 1925–26, *b;* Crozier and Stier, 1924–25, *b;* 1925–26, *b*). It seems to us inherently probable that disturbances of these types are likely to be encountered with greater frequency in objects such as isolated cell masses in culture than in connection with organs of intact animals, although we may also suggest their probable occurrence in the heart rhythms of embryos. The plottings given by Murray (1925–26) contain features suggestive in this respect, which we venture to predict will find explanation in further studies of embryonic heart rhythm.

There is one general aspect of this whole matter which requires brief additional comment. Murray (1925–26) has suggested that

the essential difference between the gross results of his observations and those in cases where intact organisms have been employed lies in the operation of some regulatory property of the complete organism. Since there is a possibility of vagueness in the understanding of such

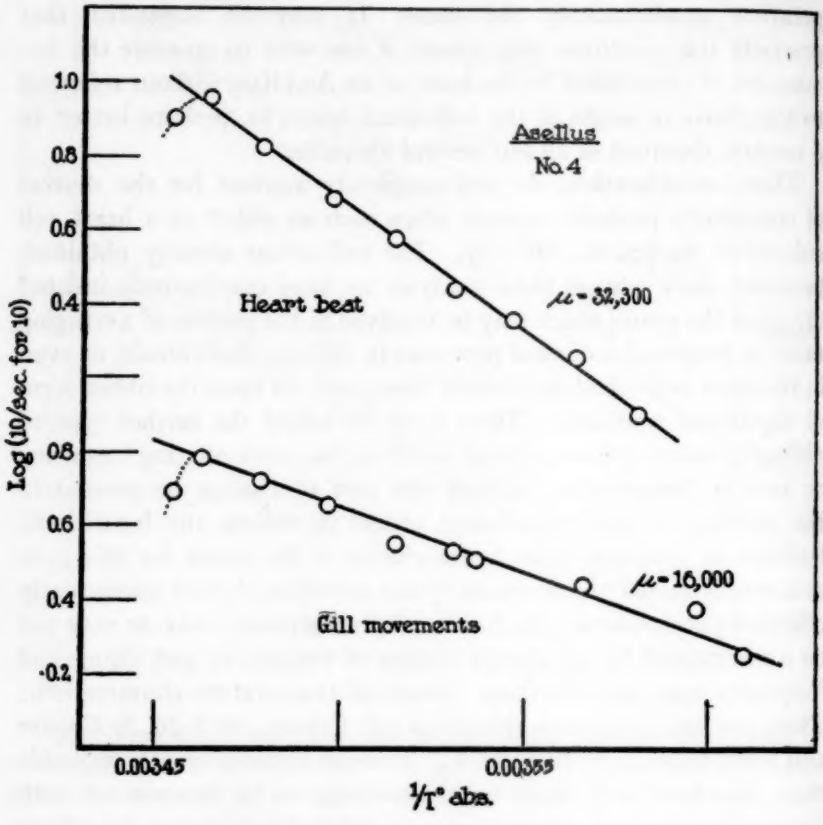

FIG. 7. Simultaneous determinations of frequency of gill movements and of heart beat in *Asellus* show that the temperature characteristic is not the same for the two activities. This disproves determination of μ by the organism as a whole.

a suggestion we may point out that it may be understood in two ways open to test. The "organization factor" might pertain simply to the heart or other structure immediately implicated in the observations; or it might be taken as a feature of the organism as a whole.

The former effect can and must be granted at once as an obvious truism, in the sense that the structure of a heart, for example, permits control by a definite localized pace maker. The latter view can be tested by determining simultaneously the temperature characteristics for two or more different activities in the same individual. We have previously made such experiments. The frequencies of heart beat and of respiratory movements in the same individual arthropod, synchronously determined, do not vary together and do not have the same temperature characteristics. This holds also for embryonic *Limulus*, the frequencies of gill movements providing increments quite different from those here obtained for the hearts (*cf.* Crozier and Stier, in a subsequent paper). For the moment we may illustrate the point by means of data from experiments with *Asellus* (Fig. 7). Therefore a general control by the whole organism is excluded. The results of these experiments will be detailed in another place. They are patently significant for the theory that a specific thermal increment has a particulate locus.

SUMMARY.

Temperature characteristics for frequency of myogenic heart beat in *Limulus* embryos, before the onset of nervous control of the heart, were found to be 11,500; 16,400; 20,000; 25,500. The two first values are the best established. The different values pertain to the hearts of different individuals outwardly similar, and to the hearts of single embryos in different parts of the temperature range. These values differ from that known in connection with the control of the heart beat through the cardiac ganglion. The occurrence of critical temperatures, also, is not the same in all embryos. These facts are employed in a discussion of temperature relations in pulsating explants of chick myocardium.

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GEOTROPIC ORIENTATION OF YOUNG RATS.

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I.

In describing the geotropic conduct of young rats (*Rattus norvegicus*)[1] we have stressed the fact that to obtain data suitable for quantitative treatment of the gravitational orientation it is necessary to employ individuals of the closest possible similarity. This has meant, in practice, employing litter mates from lines long inbred, under external conditions as nearly uniform as possible. The result has demonstrated that, with these precautions taken, it is possible to formulate precisely the connection between the intensity of excitation and the extent of the geotropic orientation. On a creeping plane inclined at angle α to the horizontal, and in which the effective gravitational component is therefore proportional to sin α, the orientation path is one defined by an angle θ on the plane, such that $\theta = K \log \sin \alpha - C$. The precision of the orientation increases according to the same equation. In addition, $- \cos \theta = K \sin \alpha$. The *speed* of progression bears similar relations to the angle α.

It was proposed to interpret these results as signifying that on an inclined plane the rat orients upward until the difference between the work done by the legs of the two sides is reduced to a certain (constant) fraction of the total. It is possible to entertain this view because the differential postures of the legs encourage it, and because they are extended in the plane of creeping. It is supported by the effects of increasing the mass lifted during creeping, as by attaching weights to the base of the animal's tail. This conception of the geotropic excitation controlling the amount of orientation as a proprioceptive matter is strengthened by further findings in this laboratory regarding the

[1] Crozier and Pincus, 1926; 1926–27, *a*, *b;* Pincus, 1926–27.

orientation of molluscs. Mr. T. J. B. Stier has also observed an identical type of orientation in newts (*Notopthalmus*), with the additional important fact that when the sign of orientation is reversed, and the animal then becomes *positively* geotropic, the angle θ is the same as in the more usual geonegative orientation.

An immediate corollary to these findings in rats of one type (*R. norvegicus*) was the possibility that certain genetic or specific differences might find expression by means of the constants in the equations for geotropic behavior. This deduction we now propose to illustrate. As material for this purpose we chose a strain of the roof rat (*Rattus rattus*). Individuals of the proper age, 13 days, were very kindly

TABLE I.

The angles of upward orientation (θ) during creeping of young *R. rattus* upon a plane inclined at angle α to the horizontal. The values of θ are each the mean of fifteen determinations, three on each of five rats in one litter.

α	θ	P. E. θ, as per cent θ
		per cent
10°	27.0°	8.74
15°	37.1°	5.78
20°	48.1°	3.68
30°	61.9°	2.34
40°	71.0°	1.88
50°	80.1°	1.72
60°	83.5°	1.06

placed at our disposal by Dr. H. W. Feldman of the Bussey Institution. We are greatly indebted to Professor W. E. Castle, and to Dr. Feldman, for this and other like assistance.

In this rat the geotropic influence could be expected to be more pronounced. Aside from the matter of its persistence into adult life, the relatively greater lengths of the legs, and the somewhat less body weight, were each expected to play a part in modifying the constants of the equations for geotropism.

II.

Five members of one litter, 13 days after birth, were employed in securing the final series of observations collected in Table I. The

experiments were made in a dark room, with temperature 20°–23°, following the technique outlined in previous papers.[1]

Within the limits of the probable errors of the means the measured values of θ adhere quite precisely to the relationship already estab-

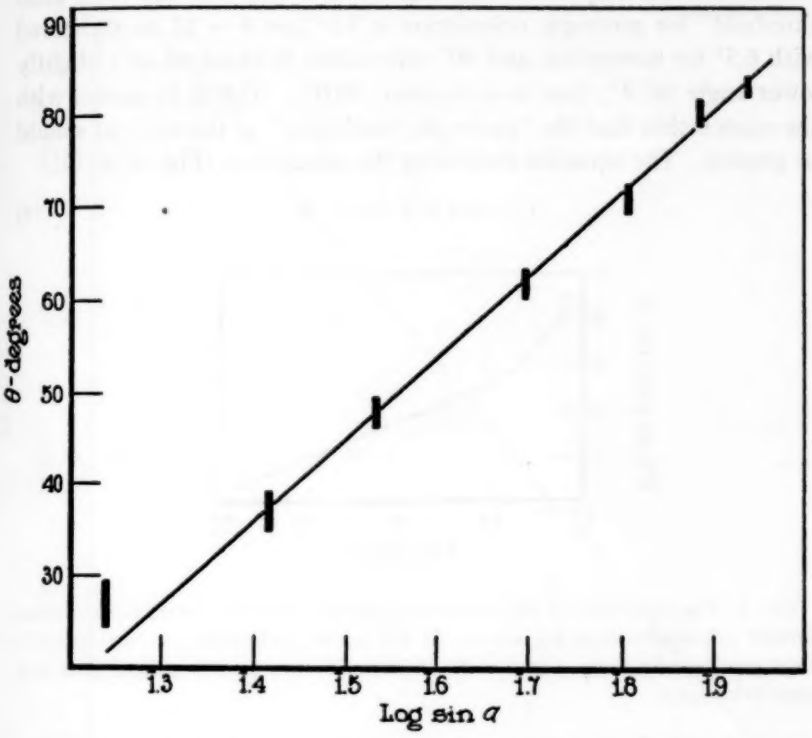

FIG. 1. The extent of upward orientation (θ) on a plane inclined at angle α to the horizontal is linearly related to log sin α during geotropic creeping of young *R. rattus*. Each observed angle of orientation is plotted as a bar of which the height = 2 P.E. The departure of the observed angle at the lowest inclination (10°) is referred to in the text.

lished[1] for the Norway rat, as Fig. 1 shows, and they thus provide an independent confirmation of it. The precision of orientation decreases linearly as log sin α increases (Fig. 2). The observations at the lowermost magnitude of α are necessarily very variable, because the slight geotropic stimulation does not sufficiently inhibit movements orig-

inating in other ways. The fact that here again, as with the Norway rat, cos θ decreases linearly as sin α increases, is made evident in Fig. 3.

To compare these results with those given by the species first used it is necessary to obtain the constants in the several equations. This is most conveniently done from Fig. 2. For the roof rat the "ideal threshold" for geotropic orientation is 3.5° [cos θ = 1], as compared with 6.5° for *norvegicus;* and 90° orientation is obtained at a slightly lower angle (67.2°) than in *norvegicus* (70.0°). This is in accord with the expectation that the "geotropic sensitivity" of the roof rat should be greater. The equation describing the orientation (Fig. 3) is

$$1 - \cos\theta = K \sin\alpha - M \qquad (1)$$

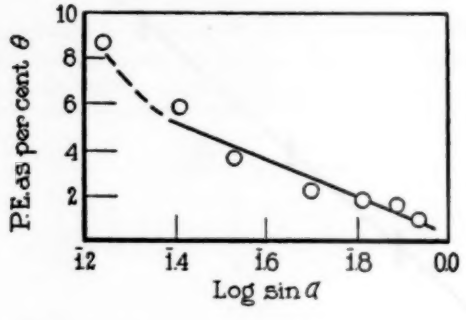

FIG. 2. The variability of the measured angle of orientation (θ) decreases almost linearly in proportion to log sin α. At the lowest inclination the variability is disproportionately large, because the "threshold" presumably differs from moment to moment.

For *R. norvegicus*, of the type used in our previous experiments,[1]

$$K = 1.206$$
$$M = 0.113$$

For *R. rattus*,

$$K = 1.18$$
$$M = 0.06$$

In terms of Fig. 1,

$$\theta = K' \log \sin\alpha - C \qquad (2)$$

For *R. norvegicus*,

$$K' = 100$$
$$C = \bar{1}.998$$

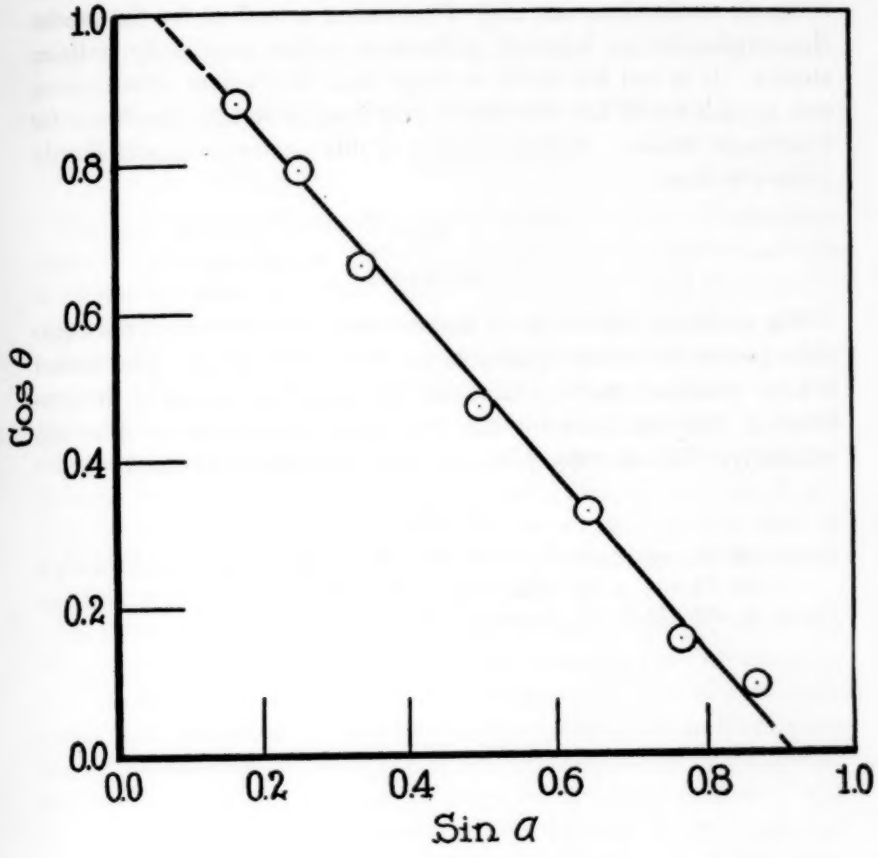

FIG. 3. As with *R. norvegicus* (Crozier and Pincus, 1926–27, *a*, *b*), in *R. rattus* the relationship between $\cos\theta$ and $\sin\alpha$ is rectilinear.

For *R. rattus*

$$K' = 90.4$$
$$C = \bar{2}.988$$

III.

Our sole purpose in recording the outcome of the present experiments is to demonstrate that the methods employed are not only capable of yielding statistically significant results in terms of an intelligible mechanism, but also of providing a method for the precise characterization of behavior differences within genetically uniform strains. It is not too much to hope that the obvious development and application of this view-point may lead to results significant for inheritance studies. Certain aspects of this matter we expect shortly to have in hand.

IV.

SUMMARY.

The geotropic orientation of *Rattus rattus* (roof rat) obeys the equations previously found applicable for *Rattus norvegicus*. The former is more sensitive, geotropically, and the numerical values of the constants in the equations for the two forms are found to differ significantly. Certain consequences of this difference are pointed out.

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GEOTROPIC CREEPING OF YOUNG RATS.

By G. PINCUS.

(From the Laboratory of General Physiology, Harvard University, Cambridge.)

(Accepted for publication, December 2, 1926.)

I.

The geotropic conduct of young rats has been discussed in a previous paper (Crozier and Pincus, 1926–27) with special reference to the angle of orientation upon an inclined plane. It was found that the angle of orientation (θ) is directly proportional to the logarithm of the gravitational component ($g \sin \alpha$) in the creeping plane. This is explicable as the result of the distribution of the pull of the animal's weight upon the legs of the two sides of the body during progression, upward orientation being the result of the "pull" of the legs on one side and the upward "push" of the legs on the other side; when orientation is attained, the ratio of the tensions on the legs of the opposite sides is regarded as constant and the difference between these tensions as a constant fraction of the total downward pull.

To examine further the nature of the geotropic conduct of young rats, observations on the speed of upward creeping were undertaken. Cole (1925–27) has discussed similar observations on *Helix;* he concludes that the speed of movement, after orientation has been attained, varies as $\sin \alpha$. But, as has been pointed out already (Crozier and Pincus, 1926–27), in these experiments the speed measured was that of *vertical* ascension, and no correction was made for the changes of θ at the different angles of inclination; such changes occur in the orientation of gasteropods. Since, at lower angles of inclination of a creeping plane (15°–70°) the animal moves at an angle (θ), it is necessary to multiply the time of upward creeping by the sine of the angle of orientation (θ) in order that the amount of time actually necessary to cover a constant distance may be dealt with at each angle of inclination (*cf.* Fig. 1). In terms of Fig. 1, the rate of creeping is given by the

fraction $\frac{AB}{t \sin \theta}$, where $AB = 32$ cm., and t is time in seconds. The required rates are therefore proportional to $\frac{1}{t \sin \theta}$.

II.

The rats used in these experiments were 13 to 14 days of age. As in the previous experiments, only animals with unopened eyes were used. To insure uniformity the animals employed were of the same age (litter mates), of known genetic constitution, and of the same weight. It was soon found that the animals show periods of activity

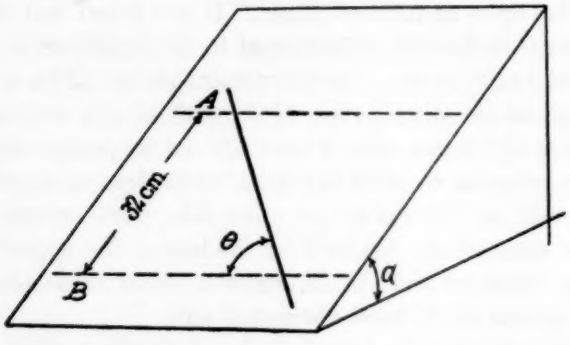

FIG. 1. On a plane inclined at angle α the path of geotropic orientation is (solid line) at angle θ; the time was measured for creeping the distance 32 cm. as indicated. The rate of progression is then obtained as $(AB)/(t \sin \theta)$.

followed by periods of inactivity, the latter being possibly due to or influenced by fatigue. Care was therefore taken to use only active individuals, and between tests each animal was allowed to rest for 20 to 30 minutes.

Observations were made in a dark room under red light of low intensity, at a temperature of 23°–25°C. The animals were placed on a creeping plane of wood covered by fine meshed copper wire. A distance of 32 cm. was marked off on the creeping plane with white chalk, and by means of a stop-watch the time was taken for the animal to creep from one white line to another.

At least ten runs were made at each angle of inclination, and at 15° and 20° inclinations, where the speed is more variable, twenty runs were recorded.

III.

Table I contains: (*a*) the times for creeping between two lines 32 cm. apart (*AB* in Fig. 1) as obtained for various angles of inclination from 15°–60°, and (*b*) the corrected rates secured by multiplying the observed rates by the sine of the angle of orientation (θ) and taking the reciprocals. No records for inclination above 60° were taken because these young animals have not the muscular equipment

TABLE I.

Angles of Orientation and Rates of Creeping at Different Inclinations of the Creeping Plane.

Angle of inclination (α)	Time to creep 32 cm. vertically	Probable error as per cent of the mean	Angle of orientation (θ)	Rates of creeping, $\frac{10}{t \sin \theta}$
15°	6.73	3.14	37.4	2.442
20°	5.42	2.94	44.5	2.636
25°	4.73	2.82	52.9	2.654
30°	4.14	2.23	57.4	2.872
35°	3.61	2.42	64.0	3.082
40°	3.40	1.84	69.8	3.139
50°	3.10	1.63	77.9	3.300
60°	2.95	1.05	84.7	3.407

necessary for uniform response to the more intense geotropic excitation of the higher angles of inclination. The speed of creeping, like the angle of orientation, varies directly as the logarithm of the angle of inclination (Fig. 2). Therefore the speed of creeping should be directly proportional to the angle of orientation (θ), and Fig. 3 shows that this is the case.

In a preceding paper (Crozier and Pincus, 1926–27) it has been shown that the *precision* of upwardly directed movement increases as the angle of inclination increases. That is, the reduction of variability (V) in the measurements of θ is proportional to the logarithm of the gravitational stimulus:

$$-V = K \log \sin \alpha.$$

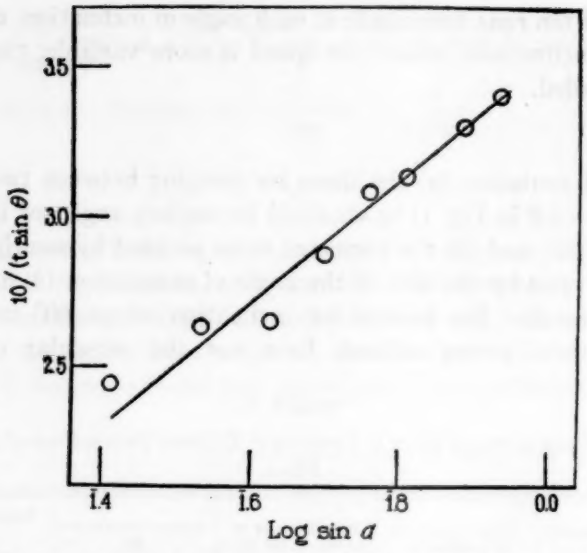

FIG. 2. The rate of creeping expressed as $10/(t \times \sin \theta)$ is plotted against the logarithm of the active gravity component. A direct proportionality is observed, the spread of the points at the lower inclinations indicating the increase in variation with lower intensities of stimulation.

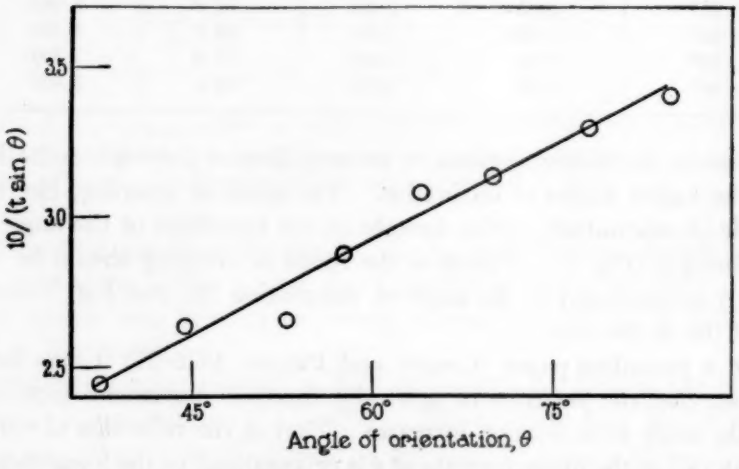

FIG. 3. The rate of creeping is plotted against the angle of orientation (θ). Since both are directly proportional to the logarithm of the active gravity component it follows that they should be directly proportional. This is the case, and a check is had upon the relation expressed in Fig. 2.

This relation is plotted in Fig. 4 for the speed of creeping. The speed of creeping is a much more complicated thing than the simple geotropic orientation, and is influenced by unanalysed fatigue effects and by cycles of activity. Furthermore, only ten observations were taken at each inclination. Nevertheless, it is apparent that the variability in speed of creeping is less at higher angles of inclination than at the lower angles, and that the relationship is linear, as in the case of the amount of upward orientation.

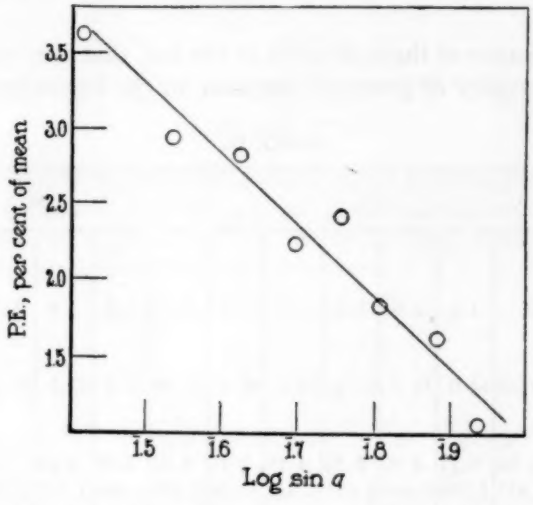

FIG. 4. The "coefficient of variation," expressed by the probable error as per cent of the mean, is plotted against the logarithm of the active gravity component. The result indicates that the variability in the rate of creeping decreases as the angle of inclination (α) is increased, and in the same manner as the variability of the extent of upward orientation (Crozier and Pincus, 1926–27).

IV.

When weights are attached at the base of the tail of a young rat creeping on an inclined plane the angle of orientation increases approximately as the logarithm of the added weight (Crozier and Pincus, 1926–27). To test this relation further, the speed of creeping with attached weights was measured at two angles of inclination, 15° and 20°. Weights of 1.6, 2.6, 5.2, 7.0, and 9.8 gm. were used.

The angle of orientation (θ) with attached weights was measured as well as the rate of creeping, and correction of the rate was made by multiplying the observed times by sin θ, as before. The results are given in Table II. Fig. 5 gives the corrected rate plotted against the logarithm of the added weight and indicates a direct proportionality for the 20° inclination; at the 15° inclination the plot is apparently curvilinear. However, when, as in Fig. 6, the corrected rates are plotted against the angles of orientation (θ) the direct proportionality observed indicates that the curvilinear distribution in Fig. 5 for 15° is accidental.

The significance of these data lies in the fact that they demonstrate the proportionality of geotropic response to the logarithm of the active gravitational component, rather than to the gravitational intensity directly. They give further confirmation of the hypothesis that the geotropic responses are the result of the pull of the animal's weight on the legs of opposite sides. This makes it unnecessary, or indeed impossible, to account for the orientations in terms of the pull of the head upon the neck muscles. It may be emphasized that as the weight of the attached load is increased, or as the angle of inclination of the creeping plane is increased, the legs are actually further extended. The angle of orientation, however, seems to be determined by the difference in effective pull on the legs of the two sides, such that, diagrammatically, at orientation $(x - y) \cos \theta = KMg \sin \alpha$, where x and y represent the lever radii of the legs on the "down" and "up" sides

TABLE II.

Angle of inclination...	15°						20°					
Attached weight (gm.).....	0	1.6	2.6	5.2	7.0	9.7	0	1.6	2.6	5.2	7.0	9.7
Angle of orientation (θ)....	37.35	44.0	51.3	67.0	72.5	84.3	47.95	51.0	62.0	78.1	80.3	90.0
Time of creeping (seconds)..	6.63	5.27	4.50	3.54	3.15	2.77	5.07	4.57	3.64	3.12	2.85	2.65
$10/t \sin \theta$....	2.477	2.734	2.849	3.071	3.332	3.628	2.658	2.816	3.115	3.276	3.562	3.773

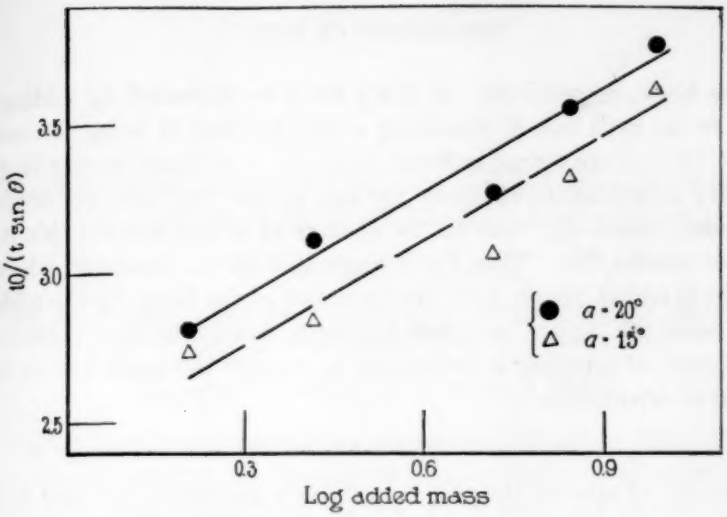

FIG. 5. The rate of creeping with attached weights at two angles of inclination (15° and 20°) is plotted against the logarithm of the attached weight. At 20° the result indicates a direct proportionality. At 15° the result is apparently curvilinear, but this may be due to fortuitous variations in θ.

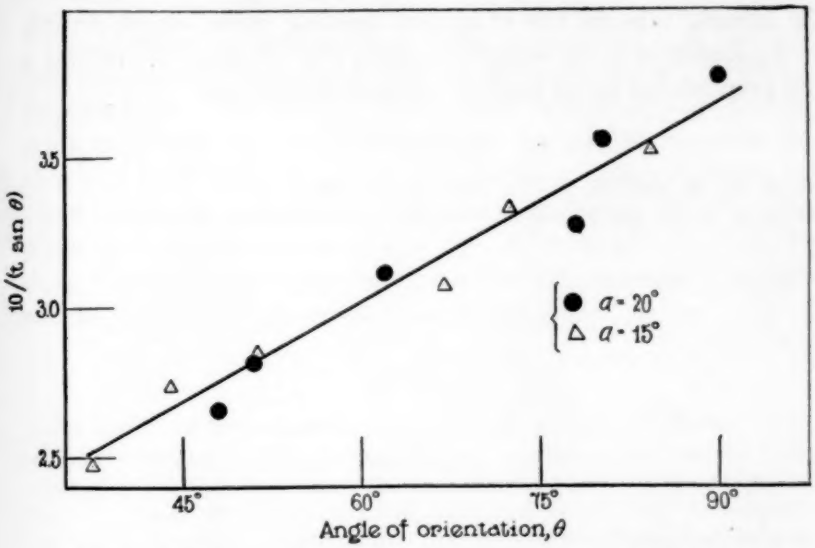

FIG. 6. The rate of creeping with attached weights at two angles of inclination (15°and 20°) is plotted against the observed angles of orientation at these angles of inclination. The result shows a direct proportionality; the curvilinear relation at 15°, as apparently indicated in Fig. 5, is not detectable.

of the body, respectively. If $KMg \sin \alpha$ be increased by adding a load to the rat's tail, K remaining a constant and M being the mass lifted, $(x - y) \cos \theta$ must increase, hence $(x - y)$ must become larger (since θ is increased); therefore the legs on the "up" side are further extended, relatively, than in the absence of added weight; this is a fact of observation. Thus the increase in θ, with α constant, when a weight is added, results from the extension of the limbs by the added load, since the "upper" or y limb is stretched and thus more extended. The speed of creeping in influenced in exactly the same way as the extent of orientation.

SUMMARY.

The rate of upward creeping in negatively geotropic rats aged 13 to 14 days is a function of the gravitational stimulus. The rate of upward movement on the creeping plane, like the angle of orientation, is directly proportional to the logarithm of the gravity component. The variability in the speed of creeping decreases in proportion to the logarithm of the gravitational effect. When weights are attached to the animals' tails the rate of upward creeping varies almost directly as the logarithm of the attached weight, and the speed of creeping is still proportional to the angle of upward orientation.

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POTENTIOMETRIC STUDIES ON INTRACELLULAR pH VALUES OF SINGLE FUNDULUS EGG CELLS.

By JOSEPH HALL BODINE.

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(Accepted for publication, December 17, 1926.)

Studies on internal reactions of cells as well as permeability of cells to acids and alkalies have usually been concerned with changes in the color of intravital indicators, introduced or taken up by the cell, as well as changes in naturally occurring cellular pigments (1–6). Death and functional changes within cells have also been used as criteria of penetration of acids and other compounds into certain specialized types of cells (2, 7). From the standpoint of an accurate quantitative treatment of intracellular pH changes in such experiments the usual errors and limitations of colorimetric as well as of mortality methods are ever present and seem to allow but limited experimental procedures. The present paper is based upon an attempt to study the intracellular reaction (pH) in single egg cells of a marine fish, *Fundulus heteroclitus*, by means of a micro hydrogen electrode and vessel originally designed for the determination of the pH values of minute quantities of insect blood (8). With suitable modifications of the electrode vessel it has been found possible to make three separate determinations on a single *Fundulus* egg cell.

Method.

The micro hydrogen electrode and vessel used in these experiments were as originally described (8) with the exception of a capillary vessel modified so that a drop as small as 0.01 cc. could be conveniently handled. Both fertilized and unfertilized eggs of *Fundulus heteroclitus*, obtained at Woods Hole, Massachusetts, were used. All eggs were taken directly from the female by "stripping" them into the solution contained in finger bowls. Eggs from each animal were kept and tested separately. In experiments where rates of penetration of acid into the cells were followed the eggs were first thoroughly washed in distilled water to free

them as far as possible of adhering electrolytes, as suggested by Loeb (9). In carrying out pH determinations individual eggs were always used and readings were made in triplicate. Between each reading the electrode was washed and checked by readings on standard buffer solutions of known pH values. The electrode vessel was at times put directly into the egg and the fluid drawn up into the capillary, while at other times small punctures into the surface of the egg with fine glass or steel needles were made and the fluid then immediately drawn up into the capillary. No significant differences were found in pH values for fluid drawn up into the capillary by these different methods. In all experiments the time consumed in drawing up the fluid into the capillary and in the taking of pH readings was extremely short, a matter of a few seconds. Eggs were individually taken from solutions by means of pipettes, washed in distilled water in the case of the acid experiments, quickly dried, and excess solution removed from the exterior by placing them on dry filter paper. They were then placed on a small watch-glass, quickly punctured, and pH determinations made on intracellular fluid. In all instances the intracellular fluid alone was used. Twenty to thirty eggs were always used in each experiment with 100 cc. of solution kept in covered finger bowls. Temperature during the entire course of the experiments ranged from 20°–22°C., but for any one experiment did not vary at any time more than ±0.5°C.

Observations and Results.

Results of typical experiments are shown graphically in Figs. 1 to 7.

Unfertilized eggs "stripped" from the female directly into distilled water or sea water give the same internal pH values, showing, as repeatedly pointed out by Loeb (9), that the eggs are little affected internally by distilled water. pH values for eggs from the same individual are fairly constant, while eggs from different individuals exhibit considerable variation—as shown in Fig. 1. These variations in eggs from different individuals are doubtless due to differences in the ages of the eggs obtained at the time of "stripping." The mean average internal pH value for unfertilized eggs at the time of "stripping" is 6.39. Unfertilized eggs kept in distilled water or in sea water and the solutions changed at frequent intervals show fairly constant internal pH values for periods up to approximately 48 hours, after which the values become progressively more acid until the egg finally dies. The most striking difference shown between a fertilized and an unfertilized egg is the marked constancy of internal pH values of the fertilized eggs as compared with the variations in the case of the unfertilized eggs (Figs. 1 and 2). This perhaps is again due to the simi-

larity in condition and age of the fertilized as compared with the various degrees of unripeness, etc., of the unfertilized eggs. Fertilized eggs show an extremely constant pH value, 6.39, even after the developing embryo has increased considerably in size (Fig. 2).

It is of some interest to note that the internal reaction of *Fundulus*

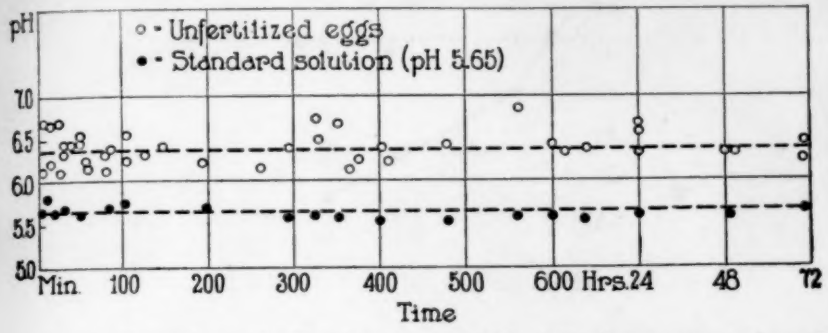

FIG. 1. Shows internal pH values for unfertilized *Fundulus* eggs at time of "stripping" from female and during exposure to sea water for different time intervals. Each point represents average of day, in some cases for eggs from same female, in others for eggs from different females.

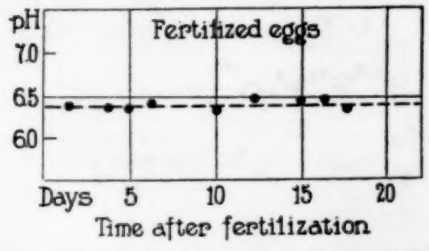

FIG. 2. Shows average internal pH values for fertilized *Fundulus* eggs during course of development of embryo. Points represent average values taken from many individual eggs.

egg, a vertebrate egg, is acid in nature despite the fact of its almost constant alkaline sea water environment (pH 8.2). Internal acid reactions, however, have been reported for various forms, protozoa, Arbacia eggs, etc. by several authors (1, 3–6).

Inasmuch as Loeb (7, 9) has so strikingly shown the extreme resistance of *Fundulus* eggs to changes in osmotic pressure, to acids,

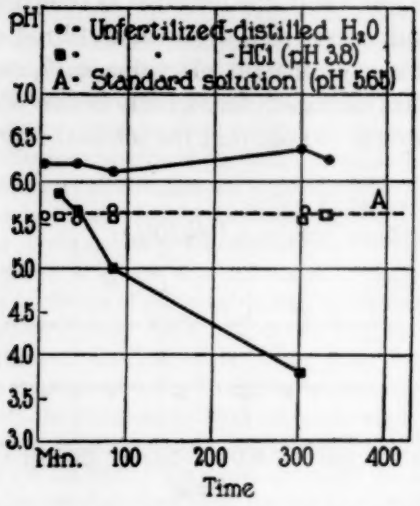

FIG. 3. Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 3.8.

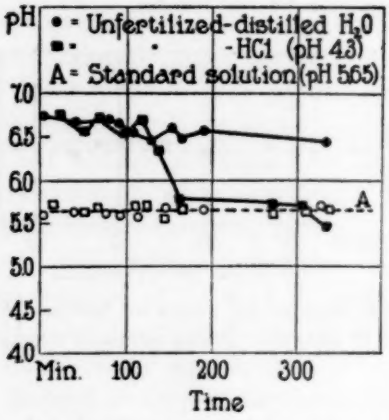

FIG. 4. Shows changes in internal pH values of freshly "stripped" unfertilized *Fundulus* eggs exposed to HCl, pH 4.3.

etc., and since by the present method the internal pH of the egg could be measured, it was thought desirable to make experiments similar to those of Loeb and to follow internal pH changes as well as func-

tional changes in the embryo. Both fertilized and unfertilized eggs were subjected to HCl of different pH values and the rates of internal

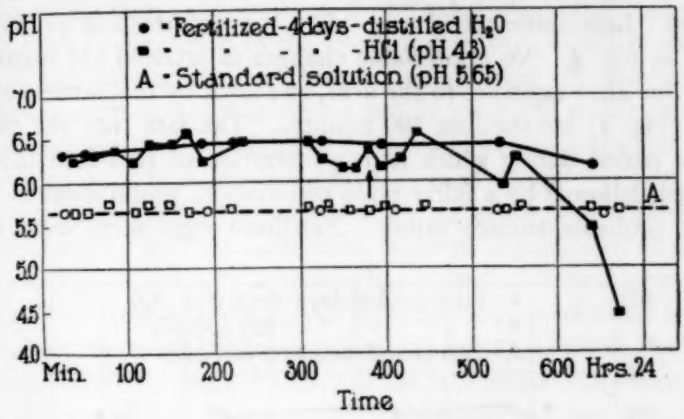

FIG. 5. Shows changes in internal pH values of *Fundulus* eggs (4 days after fertilization) exposed to HCl, pH 4.3. Arrow indicates time of cessation of heart beat and circulation.

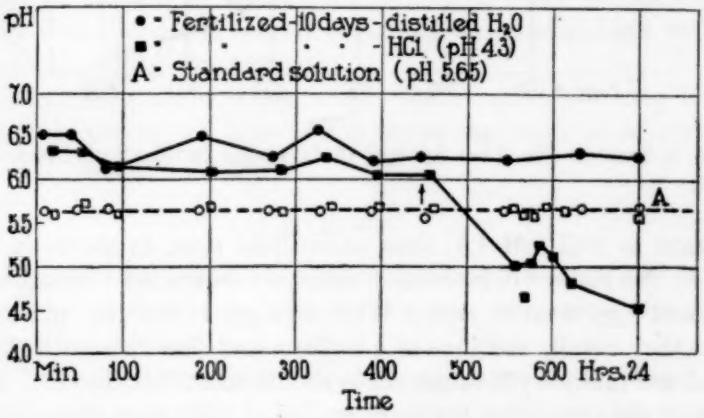

FIG. 6. Same as Fig. 5 but for eggs 10 days after fertilization. Arrow as in Fig. 5.

pH change were noted. Unfertilized eggs seem to be less resistant to HCl than fertilized eggs, as shown in Figs. 5 to 7. It is of interest

to note (Figs. 3 and 4) that HCl, pH 3.8, enters the unfertilized egg, as judged by internal pH changes, at a rapid and fairly uniform rate. Changes in opacity of the cells closely follow internal pH changes. Less concentrated acid (pH 4.3) penetrates less quickly, as shown in Fig. 4. No appreciable changes in internal pH occur for some time after exposure to the acid, as shown by the flatness of the curve (Fig. 4) for the first 100 minutes. The fact that the curves show a period during which little penetration of HCl into the egg occurred, followed by a fairly rapid penetration, would seem to indicate a probable surface injury. Fertilized eggs seem even more

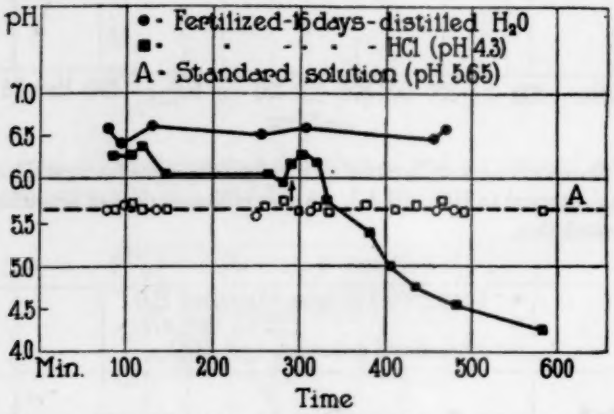

FIG. 7. Same as Fig. 5 but for eggs 16 days after fertilization. Arrow as in Fig. 5.

resistant to HCl (pH 4.3) than unfertilized eggs, as shown in Figs. 3 to 7. No marked differences in resistance correlated with age of the fertilized eggs seem to exist. When changes in internal pH values begin they usually continue at a uniform and slow rate until the external and internal pH values are in equilibrium (Figs. 3 to 7). In all cases at the time when the inner and outer pH values coincided the cell or embryo was dead. Loeb's (7) curves for the rate of penetration of HCl, pH 3.7, into the *Fundulus* egg show during the first few hours of exposure rather a uniform and gradual change in the pH of the external solutions, indicating, according to Loeb, a gradual entrance of the acid into the egg. It seems to the writer that under the conditions

of Loeb's experiments one would hardly be justified in drawing such conclusions.

In studies on the resistance of *Fundulus* eggs to acid, considerable emphasis has been given by Loeb to the assumption that cessation of heart beat or circulation in the developing embryo indicates entrance of the acid through the egg membrane. During the course of the present experiments all eggs were carefully examined under the microscope and the time of cessation of heart beat or circulation in the embryo noted. It will be found by inspection of Figs. 5 to 7, on which cessation of heart beat is indicated by an arrow, that considerable variations exist as to a correlation between cessation of heart beat and internal pH changes. As a matter of fact, in several experiments it was found that many embryos with hearts stopped could be made to recover by being returned to sea water. It seems to the author quite possible that surface effects of the HCl might easily account for such functional disturbances without internal changes resulting in the pH of the egg. It is, of course, conceivable that the delicate embryonic heart located so close to the surface of the egg might be affected by the HCl before it diffused further into the egg. Such a conception cannot be checked by the present method since we are dealing primarily with changes in the pH of the egg contents and not with the individual cells of the embryo.

These results are of a preliminary nature and show that by this method we may obtain some idea of the rates of entrance of acids like HCl into the egg of *Fundulus*.

SUMMARY.

1. By means of a micro hydrogen electrode and vessel the internal pH values of single egg cells of *Fundulus heteroclitus* have been measured.

2. Unfertilized eggs show a mean average internal pH value of 6.39. Considerable variations in pH values for unfertilized eggs exist and these are perhaps due to variations in the ages of the eggs obtained from different females.

3. Fertilized eggs show a mean average internal pH value of 6.39 with extremely small variations between eggs from different females.

4. No marked differences in pH values for fertilized eggs of different ages were detected.

5. Rates of entrance of HCl, as judged by internal pH changes, have been followed for fertilized and unfertilized eggs.

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4. Vlès, F., Reiss, P., and Vellinger, E., 1924, *Arch. phys. biol.*, iv, 21.
5. Reiss, P., 1924, *Arch. phys. biol.*, iv, 35.
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8. Bodine, J. H., and Fink, D. E., 1924–25, *J. Gen. Physiol.*, vii, 735.
9. Loeb, J., 1912, *Science*, xxxvi, 637.

THE PRECISE MEASUREMENT OF HEMOLYSIN.

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The content in hemolysin of a solution is commonly measured as the amount which is just sufficient to produce complete hemolysis of an arbitrary quantity of red blood cells, usually 0.5 cc. of a 5 per cent suspension of washed erythrocytes. The method employed for such measurement gives results which are neither exactly comparable in determinations made at different times, nor highly precise. The susceptibility of erythrocytes to hemolysis is influenced by many factors, and the amount of the minimal hemolyzing quantity must vary accordingly for every specimen of test cells. Lack of precision in the measurement results from the fact that relative differences in hemolysin content between adjacent tubes in a titration series must be great, in order to distinguish the end-point, so that the value determined differs often by a large amount from a possible true value.

The method of titration of hemolysin described in this paper was developed for a study of the association of hemolysin with different fractions of immune serum and plasma protein. It measures hemolysin content as the ratio of the hemolytic activity of a given solution to that of standard immune serum. This standard has been usually a portion of the whole serum from which isolated protein fractions have been derived.

This choice of a standard immune serum instead of a given quantity of erythrocytes, as the unit of measurement of hemolysin content, has the advantage that applies to the use of a diphtheria antitoxin for the standardization of toxin and of new antitoxin: the antibody is the most stable biological element of the immune system.

The necessity for a large increment of hemolytic substance in successive tubes in a titration to determine the minimal hemolyzing quantity has long been recognized, and is not peculiar to the immune

hemolytic system. With most, if not all hemolytic agents the increment of substance necessary to produce the final 10 or 15 per cent of complete hemolysis of a given quantity of cells is not in proportion to that which brings about the preceding fractional amount of hemolysis, but greatly exceeds its proportion. Madsen[1] made this observation first for tetanolysin, and to measure the lytic value of this hemotoxin determined the amount required to produce hemolysis equivalent to that of one-third or one-sixth of the total quantity of red cells used as reagent. Schur[2] used a similar method for the estimation of staphylolysin, and plotted the amounts of lysin against the corresponding amounts of hemoglobin liberated. The S-shaped curve so obtained is given also by serum, saponin, and NaOH, according to Handovsky,[3] although Mioni[4] had previously reported that the amount of serum hemolysis, with an excess of alexin, is proportional to the amount of sensitizer. Brooks[5] found that the amount of hemolysis is not proportional to the amount of alexin in the presence of a constant amount of sensitizer, but is represented by an S-curve which is similar to those of Handovsky,[3] and has devised a method for the titration of complement which makes it possible to compare the amounts of alexin which produce like results in constant time.

The method of titration of hemolysin content described here utilizes the same principle; it compares the amounts of unknown and of standard hemolysin which produce a definite fractional amount of hemolysis of a given specimen of erythrocytes when acting in conjunction with a given specimen of alexin.

The standard immune serum is freshly diluted for each titration and brought to a concentration such that 1.0 cc. will produce almost complete hemolysis of 0.5 cc. of a freshly prepared 5 per cent suspension of sheep erythrocytes. A series of tubes is prepared containing 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 cc. of the standard immune serum dilution. A preliminary titration of the unknown serum or fraction is carried out, if its approximate value is not known, and in a second

[1] Madsen, Th., *Z. Hyg. u. Infectionskrankh.*, 1899, xxxii, 214.
[2] Schur, H., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 89.
[3] Handovsky, H., *Arch. exp. Path. u. Pharmakol.*, 1912, lxix, 412.
[4] Mioni, G., *Ann. Inst. Pasteur*, 1905, xix, 84.
[5] Brooks, S. C., *J. Med. Research*, 1920, xli, 399.

series of three to five tubes are placed graded amounts of the unknown such that approximately 50 per cent hemolysis of the test cell quantity will be brought about by one of the intermediate tubes in the series. The fluid in all tubes of both series is brought to the same volume, 0.5 cc. of the erythrocyte suspension added with a quantitative or standardized pipette, and alexin representing two units added. For reading in the ordinary Duboscq colorimeter the final volume should be at least 4 cc. Both series of tubes are placed simultaneously into the water bath at 37°C., kept agitated, and withdrawn and placed in cold water as soon as hemolysis is complete or almost complete in the highest concentration of the standard series. After cooling the tubes are centrifugalized and the percentage amount of hemolysis in each tube of standard and unknown series is determined in the colorimeter, using the "highest" tube of the standard series as the 100 per cent standard. This is permissable since complete hemolysis of one "unit" of cells is not the end-point chosen in the comparative measurement.

A graph is prepared in which the fractional amount of hemolysis in each tube of the standard series is plotted as ordinate against the corresponding amount of diluted immune serum as abscissa. The curve is S-shaped; it varies slightly in form with each specimen of erythrocytes or alexin. From this curve is obtained the value of the abscissa of the standard corresponding to the fractional amount of hemolysis produced in each tube of the unknown series, or to the 50 per cent ordinate determined by interpolation on a curve drawn through the experimental points of the unknown.

Simple calculation then gives the ratio of the hemolysin concentration of the unknown to that of the standard. The concentration of any solution in units which produce a given fractional amount of hemolysis under the conditions of each experiment may be represented by M/V where M is the dilution, or volume in cc. in which is contained 1 cc. of the serum, or protein fraction referred to the original serum volume, and V is the volume of diluted solution which is required for the given amount of hemolysis. Then the ratio of unknown concentration to concentration of standard is given as follows:

$$\frac{M_x}{M_{std.}} \times \frac{V_{std.}}{V_x} = \frac{C_x}{C_{std.}}$$

for each fractional amount of hemolysis.

The ratios obtained from varying amounts of hemolysis have been found to agree within 4 per cent if the ordinates chosen lie between 15 and 85 per cent on the scale of ordinates so that the probable error or divergence from the mean is not greater than 2 per cent for any single reading. Consequently a single tube of dilution of unknown may be used for measurement of hemolysin concentration, if its value falls within the limits mentioned.

It is important that both alexin and erythrocytes be fresh and that the cells be washed in only two changes of isotonic suspending medium. More thorough washing of the cells leads to higher values of hemolysis in the first part of the curve, with little effect on the hemolysis of the final portion of the curve, so that greater error is introduced in determining the abscissa of the standard which corresponds to the ordinate of the unknown.

Comparison at different times, with different specimens of erythrocytes and alexin, between the same unknown solution and the same standard has given values that agree within 2 per cent.

SUMMARY.

A method is described for the measurement of hemolysin concentration, which makes possible exact comparison of results obtained at different times and with different specimens of erythrocytes and alexin; and gives precise values with an error not greater than 2 per cent.

THE PROTEIN ASSOCIATED WITH HEMOLYSIN IN RABBIT SERUM AND PLASMA.

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The immune substances or antibodies do not occur free in the blood, but are found associated with the proteins of the serum or plasma. With certain phenomena in the combination of immune body and antigen, the behavior commonly observed may be a function rather of the associated protein than of the immune body itself. "Protective" or "sensitizing" colloid effects on cell suspensions are brought about by a number of proteins which do not possess immune body.[1-4] The formation of a film of protein on the surface of the cell has been offered as an explanation of such cases.[1,2] It is not certain, however, that it is the protein nature of the film in the case of immune sera which is responsible for the changes in the physicochemical properties of the immune system. On account of the influence of "indifferent" proteins, and of protein derivatives,[4-6] even when present in low concentration, experiments planned to throw light on this problem can be undertaken only with immune body preparations that contain no protein except that intimately associated with immune body. The present investigation was undertaken in the endeavor to obtain such a protein fraction of immune serum or plasma. Hemolysin was chosen as the immune body because of the relative ease and accuracy with which this antibody may be titrated.

[1] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.
[2] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655.
[3] Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921–22, iv, 669.
[4] Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.
[5] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 395.
[6] Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538.

Hemolysin, or hemolytic sensitizer, is associated in rabbit serum with the globulin, as is immune body in general,[7] and has been found chiefly or entirely in the pseudoglobulin fraction.[8–11] Recently, however, hemolysin has been described as occurring entirely or in greater part in the euglobulin fraction.[12,13] The assignment of immune body to one fraction or another must depend on the experimental definition of the fractions of serum.

In the present work protein fractions have been obtained from the serum or plasma of rabbits immunized to sheep erythrocytes by dilution with water, and dialysis, after adjustment of the pH to the optimum for separation of the less soluble or globulin fractions. The content in hemolysin of the fractions obtained was determined by the method described in a previous paper.[14]

From serum the fraction commonly known as euglobulin was found to precipitate on dilution at an optimal value of pH 5.9 to 5.8. The amount of protein brought down and the hemolysin recovered in the reprecipitated globulin varied with the degree of dilution: the precipitate from a dilution of 1 to 5 contained 1.4 per cent and from 1 to 20 dilution contained in three sera 25, 36, and 45 per cent of the total hemolysin of the corresponding whole serum. Further lessening of the electrolyte concentration by dialysis in 1 to 6 dilution of whole serum or of the solution from which globulin had been removed by dilution alone led to the separation of a larger amount of globulin and immune body. The pH of the solutions was adjusted to 5.9; dialysis was carried out in collodion sacs for 1 to 7 days. The total amount of immune body recovered by this procedure represented 45, 28, and 62 per cent of the total present in three sera. The first value represents an increase of 9.5 per cent in terms of the con-

[7] Pick, E. P., *Beitr. chem. Physiol. u. Path.*, 1902, i, 351.

[8] Fuhrmann, F., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 417.

[9] Meyer, K., *Arch. Hyg.*, 1908, lxvii, 114.

[10] Ruppel, W. G., Ornstein, O., Carl, J., and Lasch, G., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 188.

[11] Locke, A., and Hirsch, E. F., *J. Inf. Dis.*, 1924, xxxv, 519.

[12] Otto and Sukeninkowa, *Z. Hyg. u. Infectionskrankh.*, 1924, ci, 398.

[13] Laubenheimer, K., and Vollmar, H., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 202.

[14] Coulter, C. B., *J. Gen. Physiol.*, 1926–27, x, 541.

tent of the whole serum over that obtained by dilution to 1 to 20 alone, at pH 5.8.

This globulin precipitated most promptly from aqueous solution at pH 5.65. No fractionation of the protein was possible by variation of the pH of aqueous solutions. If, however, NaCl in substance was added to the concentration of isotonicity to an aqueous solution of the globulin, a precipitate was obtained which varied in amount and in time of appearance in different specimens and formed in a rather wide range of pH with an optimum near pH 5.0. It was often incompletely soluble in water at pH 7.0 to 8.0.

Preparations from two sera had a hemolysin content of 0.15 and 0.63 per cent of that of the corresponding whole serum. The hemolytic activity of the globulin solution after the separation of the salt-insoluble precipitate was found to be 1, 5, and 12 per cent greater than before its removal. The salt-insoluble fraction appears thus to act as antialexin; this property is destroyed by heating to 50°C. The nature of this fraction is uncertain. It was at first regarded as a denaturation product, as described by Wu and Yen,[15] as a result of the hydrion concentrations to which the serum had been subjected. Later work with plasma suggested that it may be residual fibrinogen.

The hemolysin content of the solution which remained after dialysis was similar to that obtained with plasma, which is described below.

In obtaining plasma from immunized rabbits, coagulation was prevented by sodium citrate or potassium oxalate. On dilution of plasma with water, a flocculent precipitate consisting mainly of fibrinogen appeared at the optimal reaction of pH 6.4 to 6.1.

This fraction from a 1 to 5 dilution contained 2.3 and 2.5 per cent, and from 1 to 10 dilution 2.6, 5.5, and 6.5 per cent of the hemolysin of the corresponding whole plasma. In carrying out the titrations of solutions containing fibrinogen it was necessary to make the initial dilutions with water, to prevent fibrin formation. The fibrinogen itself in this fraction appears not to carry immune body: the hemolytic titre after the separation of fibrin by initial dilution in saline solution was the same within the limit of error in measurement, as

[15] Wu, H., and Yen, D., *J. Biochem.*, 1924–25, iv, 345.

when clotting was prevented by initial dilution in water. Furthermore, the presence of fibrinogen under certain conditions depresses the apparent hemolytic activity of the immune protein, as found and described below, with the second fraction of plasma.

Globulin and a second portion of fibrinogen were separated from the remaining plasma solution by dialysis at pH 6.1 against distilled water. Thymol was added, and dialysis carried out at 5°–10°C. for 3 to 15 days. The greater portion of water-insoluble protein flocculated in 48 hours; a further small amount, which was identical in its solubility with the first portion, separated slowly during 15 days or more. The precipitate, which appeared on dialysis, was soluble in water, and showed an optimum for flocculation from aqueous solution at pH 6.1 An hemolysin content of 33, 48.5, 48, 62, and 68 per cent of that of the whole plasma was obtained in this fraction. Longer dialysis was employed with the later experiments, with resulting increase in separation of hemolysin as indicated by the values which are given in the order in which the experiments were carried out.

The fibrinogen present in this fraction separated out as fibrin when NaCl was added to isotonicity to an aqueous solution. Fibrin formation took place at reactions between pH 8.0 and 5.6, with an optimum about pH 7.2; separation was slow and three or more clottings were observed if each clot was removed as it formed. Within the limits of pH given, the formation of fibrin gel was most rapid and complete within certain concentrations of NaCl or $CaCl_2$; the limits were not precisely determined, but approximated isotonicity for NaCl. On the acid side of pH 6.0 fibrinogen separated out as a granular precipitate which was greatest in amount at pH 5.0 to 4.8. Fractions of immune protein which contained fibrinogen showed the greatest hemolytic activity, when brought in saline solution to pH between 8.0 and 4.0, at the reactions optimal for formation of fibrin gel. The presence of fibrinogen, possibly because of the form in which it existed, in solutions at other reactions appears to depress the hemolytic activity.

The total N associated with one hemolytic unit, of the usual value, was found with globulin preparations freed from fibrinogen to be 0.00038, 0.00034, and 0.00007 mg. with three different specimens.

The values are very close to those of Locke and Hirsch[11] for hemolysin obtained by dissociation from specific combination.

The hemolysin present in the plasma solution from which water-insoluble globulin had been separated by dialysis varied inversely with the amount recovered in the globulin fraction; a minimum of 2.8 per cent of the total was found in a specimen which had been dialyzed for 15 days.

DISCUSSION.

The globulin with which hemolysin is associated appears to exist in plasma as an adsorption complex with fibrinogen; the latter determines the optimal pH of flocculation from aqueous solution of the complex. The conditions under which fibrinogen forms a fibrin clot, or gel, recall those found by Falk[16] for gel formation by banana protein; in both cases gel forms only on the alkaline side of pH 6.0 and within certain limits of salt concentration. The relation of Ca to fibrin formation in the solutions studied is not as evident as in the case of banana protein; Ca is known to be necessary for clotting only in the first step, for which blood Ca was available in these plasma preparations.

The granular form in which fibrinogen separates from isotonic NaCl solution at pH 5.0 to 4.8 suggests that a similar precipitate from serum protein solutions is residual fibrinogen. Both fibrinogen and the salt-insoluble protein from serum carry down no hemolysin or only an insignificant amount which may represent adsorbed globulin. The depression of hemolytic activity by amorphous precipitate of fibrinogen is perhaps due to adsorption of alexin.

Extraction of immune globulin preparations, with 8 to 20 volumes of 95 per cent alcohol at 0°C., caused a loss of at least 90 per cent of the immune body. The evaporated residue of the alcoholic extract contained both protein and lipoid, which neither alone nor together showed hemolytic activity. At the same time, the hemagglutinating property of the immune protein was found considerably intensified after alcoholic extraction. The partial denaturation of the protein by alcohol destroys the hemolytic activity, which must depend upon a different property of the protein from its agglutinating action.

[16] McGuire, G., and Falk, K. G., *J. Gen. Physiol.*, 1921–22, iv, 437.

The water-insoluble globulin which was obtained by dialysis was found to contain both euglobulin and pseudoglobulin, defined as fractions precipitated by one-third and one-half saturation with ammonium sulfate. Although the separation of pseudoglobulin and immune body from serum or plasma by dialysis at optimal pH was not complete, the present work confirms the view of Pauli and Adolf[17–19] that no distinction can be made between a water-soluble and a water-insoluble globulin.

SUMMARY.

1. The water-insoluble globulin with which hemolysin is associated, may be separated from immune serum or plasma by dilution and simple dialysis at optimal pH.

2. This optimum in plasma is influenced by the presence of the fibrinogen.

3. Fibrinogen carries no immune body, or only an insignificant amount; when present in immune body solutions in other form than fibrin gel, it depresses the hemolytic activity. The conditions for the formation of fibrin gel are similar to those for the formation of a gel by banana protein.

4. The hemolytic activity is a more labile property of the immune protein than the agglutinating activity; hemolysin is destroyed, hemagglutinin shows an apparent increase, as a result of alcohol extraction.

[17] Pauli, W., *Biochem. Z.*, 1924, clii, 355.
[18] Adolf, M., and Pauli, W., *Biochem. Z.*, 1924, clii, 360.
[19] Adolf, M., *Klin. Woch.*, 1924, iii, 1214.

"GALVANOTROPISM" OF ROOTS.

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I.

The galvanotropic orientation of animals may probably be used for analysis of certain features of central nervous activity.[1] Therefore it is necessary to obtain a conception of the mode of stimulation by passage of direct current. In relation especially to the results of studies, in this laboratory[2] and elsewhere, upon the conductance of plant cells, and because of the structurally simpler conditions of response, we have paid attention to the well known "galvanotropic curvature" of roots.

Every one interested in plant irritability has always considered the "galvanotropic response" of the roots as one of the most remarkable facts described. Discovered by Elfving in 1882, the "galvanotropic response" was studied by Müller-Hettlingen, 1883; Brunchorst, 1884; Rischawi, 1885; Ewart and Bayliss, 1906; Schellenberg, 1906; and in a rather long paper by Gassner, 1906. Indications are given also by Szücs, 1913, and a general review of the subject can be found in Stern's book (1924).

Based on all these observations, it is generally admitted that for high densities of current, or for long exposures, a curvature towards the + pole is obtained (so called Elfving's curvature); that, on the other hand, for lower densities of current, or for shorter exposures,

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[1] *CF.* Crozier, W. J., 1926, *J. Gen. Physiol.*, ix, 395.

[2] Osterhout, W. J. V., 1926, *J. Gen. Physiol.*, viii, 131. Blinks, L. R., 1926, The permeability and electrical conductivity of single cells, Thesis, Harvard University, Cambridge, 1926.

the curvature is directed toward the − pole (genuine "galvanotropic response"). This last is considered as a tropism, the first curvature being said to be purely traumatic.

The definition of the true "galvanotropism" of the root can be therefore expressed in the following manner: It is a growth curvature, directed towards the cathode, located in the region of maximum growth, irreversible by plasmolysis and requiring the presence of the tip of the root.

The origin of this curvature is naturally the real question. For the explanation of this origin several theories have been advanced. Brunchorst sees in the curvature an injury by the electrolysis products, especially the H_2O_2 that may be produced. Rischawi considers the phenomenon similar to the electrosmotic water displacement in the albumin cylinders of du Bois Reymond's experiments. Ewart and Bayliss attribute the response to chemotropic stimulation by the products of electrolysis, all idea of traumatic curvature being excluded.

On the contrary, for Gassner the facts may be explained as a traumatic response of the organism to a unilateral injury of the tip of the root.

In fact, to all these explanations the same objection may be made: the technical conditions were often too crude and, in a certain number of cases, too vague to be used adequately as bases for explanations. For instance, Ewart and Bayliss used platinum electrodes directly in contact with the plant. In other cases, it is true, so called unpolarizable electrodes were employed. Others (Brunchorst, Gassner, Schellenberg) used carbon electrodes, often dipping directly in the same liquid as the root tips but sometimes surrounded by a septum made by a porous plate. For many cases the density of current corresponding to the position of the roots in the trough is not known.

It was thought interesting for these reasons to reproduce these experiments, trying always to avoid the errors pointed out.

The actual experiments were carried out keeping in mind the following points: (1) Reduction of polarization products by use of unpolarizable electrodes, (2) prevention of diffusion of the products of electrolysis by use of agar blocks, (3) gradient of densities of current easily controllable by use of troughs with definite geometrical shapes and sizes.

II.

Technique.

1. Trough 1.—Paraffine blocks were carved out following the indications of Fig. 1. These troughs are characterized by their variable cross-section, one end being a square of 5 cm. of side, the other end being 2 cm. × 5 cm. The distance of these two sections is 25 cm. and each section is closed by a block of agar gel. The agar was purified agar which had been soaked in 2 per cent HCl for 24 hours; then in 1 per cent ammonia for 12 hours; then subjected to running water for 48 hours, all with frequent shaking. After this treatment the water was more or less pressed out and the agar washed several times with distilled water, this being also used to make the gel, of which the concentration was 10 per cent in dry weight of agar. This agar gel is

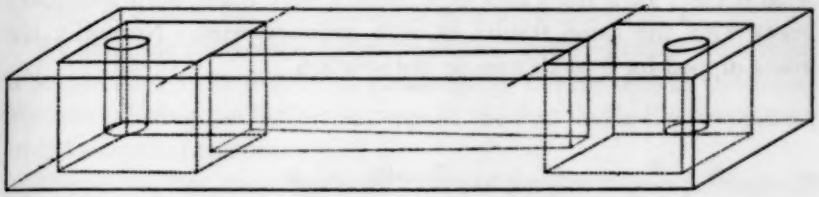

FIG. 1. The figure represents Trough 1. In Trough 2 the blocks of agar are omitted and replaced by porous plates in the vertical planes of the dot-dashed lines.

poured in place, care being taken to obtain a plane surface at both ends of the trough (the real electrode surfaces) and cups being provided in each block for the electrodes. These cups as well as the trough are filled to a definite height with tap water or with a balanced salt solution (diluted Knop solution). This liquid was removed immediately after each experiment and the trough was washed for several hours by a continual flow of tap water.

2. Trough 2.—The other type of trough is similar, the differences being only in the replacement of the agar blocks by porous plates 2 mm. thick.

3. Electrodes.—The electrodes used were carbon cylinders 2.5 cm. in diameter, a good contact being assured for the leads; or unpolarizable electrodes, either calomel electrodes (employed in a few cases only,

the inner resistance being too high) or of zinc-zinc sulfate. In this last case the electrodes, which were kept in a concentrate solution of zinc sulfate, were rapidly washed before use under running tap water. Furthermore, care was taken to have the same hydrostatic level in cups and electrode.

4. Current.—The potential differences applied to the electrodes varied from 0.5 volts to 115 volts. The density of current (according to the position of the root in the trough) varied from 0.058 ma./sq. cm. to 1 ma./sq. cm. The time of exposure to the action of the current was changed between 15 minutes and 360 minutes.

5. Material.—The plants used were *Vicia faba* (broad Windsor beans) and *Phaseolus vulgaris* (Burpee's improved bush lima beans). The seedlings were grown in sawdust at 20°C. and their roots were practically straight. They were used when the length of the roots was 4 to 6 cm. In a few cases experiments were made with secondary roots with the same results as with primary ones. Normally the roots dipped for 8 to 10 mm. in the solution.

III.

Results.

1. When Trough 1 is used alone, whatever the P.D. or density of current may be, and whatever the duration of the experiment (between 15 minutes and 360 minutes) no curvature ever occurs.

When Trough 2 is used, with the same conditions, curvatures are shown if carbon electrodes are used and always towards the cathode.

A test was to put Troughs 1 and 2 in series, as the objection could be made that the density or time was deficient: under these conditions curvature appears in No. 2; no curvature in No. 1, so the current and time of exposure were large enough to produce "galvanotropic" responses.

The immediate conclusion to be drawn from these experiments is that "galvanotropic" curvature is produced by the products of electrolysis.

2. Was therefore the galvanic current necessary? Sets of seedlings were put with the root dipping for 1 cm. in the water of Trough 2, for 24 hours, after the current had been passed through the water for

2 and 4 hours. Care was taken not to move or disturb the water during or after the electrolysis.

The immediate result of this test was to show a slight but definite curvature—in fact less than when the current was acting directly on the seedlings and practically not directed toward one pole or the other. The angle of deflection of the tip of the root was about 15° to 20°.

If the same experiment was made, with the same conditions (carbon electrodes, 2 or 4 hours previous passage of current) with Trough 1, no curvature is shown.

This fact proves that the blocks of agar, 10 cm. in length, are sufficient to prevent the diffusion of products of electrolysis or to slow it up so that they do not reach the middle part of the trough in time to affect the seeds.

There is therefore a difference in the curvatures when current is present or absent but the difference is merely quantitative.

We must for this reason recognize in the "galvanotropic" curvature a double effect: the first being produced by the electrolysis products; the second being the further increase of the first under the persistence of the electrical current.

The second test, showing the influence of the electrolysis products is, in fact, sufficient to show that the primary effect on the root is a traumatic one.

3. Another way to show this was to injure the root before the experiment and to place it then in Trough 1. If really injury is the first step, curvature must occur under these conditions, with non-polarizable electrodes. And it does.

Roots were placed for 2 to 3 minutes in a solution of copper nitrate N/100; then in Trough 1 filled with tap water. The current was passed for 60 minutes. The plants were left in place in the same liquid for 24 hours (as in all the other experiments) after the passage of the current. At the end of this time a definite cathodic curvature was shown.

Experiments made after immersion of the roots for 10 minutes in the same copper solution did not give curvature.

Microchemically it is easy to show that during the short exposure the two external layers of cells are permeated by the copper ion, and

that in the second case a much larger number of layers are injured. The latter injury corresponds practically to the killing of the root.

So, to produce the "galvanotropic" curvature in the absence of electrolysis products, the injury must be definite but not too large. A certain amount of tissue must remain in the root to react. The curvature is in fact a response from injured but not dead tissues.

IV.

Interpretation.

How may we try to explain these facts? As was pointed out previously the response must surely be developed in two steps (or more).

Let us consider a root dipping in the water between the two electrodes. This body in the electrical field naturally repells the lines of flow around itself, as we know that the living cells are practically nonconducting for direct current. But under these conditions there may be accumulation on the opposite sides of ions of opposite signs. And this may be sufficient to injure the epidermic layer of the root. What are the ions which are so toxic? It may be said that perhaps any ion present in the solution used will act in this way.

In a root of circular cross-section we may thus consider two opposite halves in the epidermic layer, both injured and probably with a number of the cells killed, and acting for the remaining part as two electrodes directly applied on the internal tissues. These electrodes determine, inside of the root, an electrical field, the conducting paths being made by the cellulosic membranes imbibed with aqueous solutions. The resulting electrolysis acts now on more deeply situated cells.

The produced ions are formed in such loci that they may act directly on the plasmatic surfaces, inducing in these such changes that the relative dielectrical resistance diminishes and that free ions may migrate in or out of each cell. The result is necessarily that under the directing action of the electrical field, in all cells through the root, chains of + and − charges are formed, each cell having a + charge on the cathodic side and a − charge on the anodic side. The perduration of the current continues the same action in the same way and causes finally a relative increase of anions on the anodic side and of

cations on the cathodic side. The two halves of the root are brought, by this process, to be ionically different. The early effect of the anions on the anodic side seems not to prevent growth. The relative accumulation of cations, on the contrary, slows the growth on the cathodic side, and by further increase stops it completely. The effect of this differential state is a bending towards the cathode.

V.

In the earlier papers on galvanotropism another type of curvature has been described, the so called Elfving's or anodic curvature. With the device described in the present paper it has always been impossible to obtain this effect, even when the root was deeply dipping in the water. We may draw from this fact the deduction that in the cases described, with very high densities of current, other factors were interfering.

The same can be said as to the *S*-shaped curvature described by Gassner when more than the tip of the root dips into the liquid: in the present conditions of experimentation no case of this bending was found. Is it therefore to be deducted that this type of curvature does not exist? No, probably; but that under the conditions of experimentation, insufficiently described in the papers to which reference is made, other effects are occurring. Other experiments are necessary to ascertain the conditions of production of these curvatures, as well as the change in conductance of the tissues showing or not showing these reported curvatures.

SUMMARY.

1. New experiments, made in such a way to eliminate as completely as possible products of polarization and the migration of such products when formed, have shown that the exhibition of galvanotropic curvature in roots is mainly dependent upon such products, since no curvature appears when they are excluded.

2. The polarization products injure the external layer of cells of the root; this allows these cells to act as electrodes directly applied on the internal tissues. The inner electrolysis produces such changes in the interior cells that they may be considered as becoming ionically different. This differential state is responsible for curvature.

3. "Galvanotropism" of roots, therefore, cannot be regarded as exactly comparable to the galvanotropic orientations of certain animals, but is essentially dependent upon injury.

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THE FREE ENERGY OF NITROGEN FIXATION BY LIVING FORMS.

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The view-point that energy is required for the fixation of nitrogen by the various forms of life seems to be generally taken for granted among biologists.

It is the purpose of this paper to present evidence of a simple thermodynamic nature against the soundness of this current conception. It will maintain that, contrary to the current view, energy may not be required for fixation, and that moreover, in fixation considerable energy may be released and placed at the disposal of the organism. In contending for a position so plainly the reverse of the old, important thermal data will be used which has been obtained only in the last decade.

This paper, as so many others, is indebted to Lewis and Randall[1] for most of the free energy values and conventions to be used. Unless it is otherwise stated, the temperature of all the reactions cited is 25°C. The heat of reaction and the free energy of reaction will be negative when heat and free energy respectively are liberated.

Nitrogen fixation may be defined as the primary step or steps in the formation of a simple nitrogen compound such as ammonia or nitrate from pure elementary nitrogen. It is to be distinguished from later changes in which ammonia and nitrate are formed one from the other, and also from the building up of higher compounds with carbon. When an organism once forms a simple compound from elementary nitrogen, the energy relations are those which would exist were it to be supplied with such a simple compound and we must discriminate between the energy of nitrogen fixation and that of nitrogen metabolism.

[1] Lewis, G. N., and Randall, M., Thermodynamics, New York, 1st edition, 1923.

The standard free energy, ΔF°, of the reaction

$$1/2\ N_2(\text{gas}) + 5/4\ O_2(\text{gas}) + 1/2\ H_2O(\text{liquid}) = HNO_3(\text{aqueous})$$

is a small positive value, showing that at standard conditions this reaction is not spontaneous and will proceed only with the addition of free energy. At standard conditions all the reacting substances have unit activity; that is to say, the gases have fugacities of one atmosphere (for the present calculations their pressures may be considered equal to their fugacities), the water is pure, liquid, and at atmospheric pressure, and the concentration of the nitric acid is at the hypothetical 1 molal. Let us now calculate the conditions in the plant when the above reaction will proceed without the addition of free energy, that is to say, when the free energy, ΔF, will be negative. Avoiding unwieldy overrefinements, let us consider as appropriate approximations, the pressure of nitrogen, .8 atmosphere; the pressure of oxygen, .2 atmosphere; the plant sap to have the same activity as pure water; and the activity of the nitric acid formed to be unaffected by the various foreign substances in the sap.

The standard molal free energies of nitrogen, oxygen, water, and nitric acid are respectively 0,0, −56,560, and −26,500 (calories). Hence in this reaction ΔF° is 1780. Now,

$$\Delta F^\circ = -RT\ ln\ K = -1364.9 \log K$$

$$\log K = \log (HNO_3\ (aq.)\ /\ (N_2)^{\frac{1}{2}} (O_2)^{\frac{1}{4}}) = -1780/1364.9 = -1.304.\quad \text{Or, } K = .0497$$

$$\text{activity } HNO_3 = K \times (.8)^{\frac{1}{2}} \times (.2)^{\frac{1}{4}} = .0067$$

From the table containing the activities of nitric acid at various concentrations this value corresponds to a concentration of just .1 M. This means that, assuming the plant can catalyze the reaction, nitrate will form in the plant with the liberation of free energy so long as its concentration remains below .1 M or 6200 parts per million by weight. At this point ΔF no longer has a negative sign but is equal to zero, since this is the equilibrium concentration.

The heat of the reaction is $-49{,}100 - (-68{,}310/2) = -14{,}940$. Owing to the fact that the plant sap rarely if ever reaches a concentration of 6200 ppm., for every mol of nitrate which forms from air and water according to the above equation there are 14,940 calories of heat (not work) liberated for partial use as chemical energy. The

more dilute the concentration at which the nitrate is formed the greater is the amount of available work (not heat) placed at the plant's disposal. Since nitrate in the plant sap is removed soon after its appearance, its concentration probably never rises much above, let us say, .001 M. If we assume a maximum concentration (*i.e.* stationary state) of nitrate in the plant sap of 6 ppm. or .0001 M, and that the activity coefficient at this concentration is unity, then the following calculations show that the free energy is −7870 per mol of nitrate formed.

$$K = (.0001)^2 / (.8)^{\frac{1}{2}}(.2)^{\frac{1}{2}} = 8.6 \times 10^{-4}.$$
$$\Delta F = \Delta F^\circ + RT \ln K = 1780 + 1364.9\,(-7.07) = -7870.$$

Similar calculations show that after making the permissible assumption that the heat of the reaction would not change significantly under conditions of such extreme dilution, the free energy of the reaction would equal the heat of the reaction, −14,940, that is to say, the process would be 100 per cent efficient, when the concentration of nitrate was 7×10^{-6} M or .4 ppm. In the sap of some plants this is the highest concentration to be found. Indeed, in studies by Hoagland and Davis with the sap of *Nitella* cells it was found that, although the pond water in which *Nitella* was growing contained about .5 ppm. of nitrate, the cell sap contained no detectable amount. If this small concentration of nitrate results from the rapid use made of nitrate by the cell, it is evidence of how efficiently the fixation of nitrogen may be accomplished. Comparison of the free energy (−7870) at a nitrate concentration of 6 ppm. with the free energy (−14,940) at the lower concentration of only .4 ppm. shows how quickly the free energy increases as the concentration of nitrate decreases.

A citation from Lewis and Randall[2] will illustrate the significance of this reaction. "It is to be hoped that nature will not discover a catalyst for this reaction, which would permit all of the oxygen and part of the nitrogen of the air to turn the oceans into dilute nitric acid."

Although foreign to the discussion of nitrogen fixation, it might be remarked that the energy needed for the reduction of nitrates following their fixation may be supplied by radiant energy in the case

[2] Lewis and Randall, p. 568.

of higher plants. In leguminous plants, should the nitrogen-fixing bacteria produce nitrates, they might release these into the sap where they could be carried to the leaves and reduced by sunlight at no energy expense to the organism.

It will be noticed in the foregoing equation that oxygen gas is required as a reactant. Does the equation for this reason fail to serve for anaerobic organisms, such as *Clostridium*, which can fix nitrogen in the absence of oxygen? We may escape this conclusion by postulating the formation of oxygen gas incidentally to some other metabolic process; a feasible supposition in absence of data known to preclude a small but continuous production of this gas during fixation. A continuous and restricted production of the gas may occur and yet escape detection, owing to the immediate use made of it by the organism. The fact that, if such a source be supposed, the activity of the oxygen in the equilibrium constant would be reduced, would affect the conclusions at issue only as regards quantities involved.

Against the value of this possible source of oxygen as support for the main contention of this paper, it may be objected that, assuming the production of oxygen gas under anaerobic conditions, would not energy be required for its production, and so leave on hand the same occasion as before for assuming an energy requirement for the fixation of nitrogen by anaerobes?

Admittedly, an examination of the score of available free energy data of organic compounds conclusively suggests energy requirement for the production of oxygen. A striking and obvious case is found in the reaction of formic acid to yield oxygen and formaldehyde, the standard free energy having a positive value of about 60,000 calories, an enormous amount when considered relatively to the free energies of either of the compounds, which are, respectively, $-87{,}920$ and $-30{,}000$ (approximately). And in general this behavior might be expected from most if not all organic compounds. However, there might be exceptions, since such inorganic exceptions can be found. In the reaction of $HBrO_3$ to yield HBrO and O_2, 14,280 calories of heat are liberated and the standard free energy amounts to $-21{,}980$; and there are several inorganic reactions similar to this one, which is given merely as an example of possibility but not with a view to application. Hydrogen peroxide is another example and the yield

of free energy is even larger than in the case just cited. There might be organic peroxides with a similar behavior.

However, in anaerobic processes oxygen need not be considered. The production of aqueous ammonia from nitrogen and hydrogen is attended by a negative standard free energy change of 6300 calories and a negative heat of reaction of 20,300 calories. This exothermic and free energy-yielding production of ammonia resembles that of nitric acid, and a similar correspondence holds between the effects which result from the formation and removal at high dilution of the two compounds ammonia and nitric acid.

Out of curiosity let us glance at the standard free energy which would be yielded if oxygen were present also. If the reaction proceeded according to

$$1/2\ N_2(g.) + 5/2\ H_2(g.) + 1/2\ O_2(g.) = NH_4OH(aq.)$$

rather than in the manner above,

$$1/2\ N_2(g.) + 3/2\ H_2(g.) + H_2O(l) = NH_4OH(aq.)$$

the standard free energy would be $-62{,}860$ as against the former -6300. This is a great increase, owing for the most part to the fact that oxygen of water as a reactant is replaced by oxygen gas. In the same way in the aerobic fixation of nitric acid if hydrogen were present making the reaction proceed

$$1/2\ N_2(g.) + 1/2\ H_2(g.) + 3/2\ O_2(g.) = HNO_3\ (aq.);\ \Delta F^\circ\ -26{,}500;\ \Delta H\ -49{,}100;$$

then a great deal more free energy would be evolved than in the original case discussed.

In these reactions the need for hydrogen production might be satisfied from several independent sources with no expense of free energy by the organism.

In anaerobic processes hydrogen is often an end-product incidental to metabolism, and would waste if not conserved in fixation.

In both aerobic and anaerobic metabolism many reactions, more particularly the breakdown of organic acids, may proceed spontaneously to yield both hydrogen and energy. For example,

$$HCOOH(aq.) = CO_2(g.) + H_2(g.);\ \Delta F^\circ = -6340.$$

It may be argued that the organism sustains a net potential if not actual loss of energy when the reaction proceeds in this manner, rather than by combustion, when 61,000 calories of heat are yielded; whereas in the reaction cited the heat of the reaction is approximately zero. To this objection it is enough to answer that if no oxygen were present this energy could not be yielded. But assuming an occurrence of oxygen, then since the heat of combustion is less by some 40,000 calories than the heat of formation (−101,000) there results a veritable gain of energy upon replacement of the HCOOH molecule. To object that the HCOOH molecule is not replaced under certain conditions is unwittingly to concede that under these conditions HCOOH is a waste product. Were HCOOH derived from glucose and oxygen gas, instead of from its elements, the difference between the heats of formation of $\frac{1}{6}$ mol of glucose and 1 mol of HCOOH; that is to say, the veritable gain of energy, is still larger than the value 40,000 calories by 10,000 calories. And if the glucose were derived from carbon dioxide and water, instead of from its elements, then an enormous amount of energy is required for fixation providing that (1) it is not fixed as HNO_3, (2) it is fixed as ammonia, the hydrogen coming solely from the glucose (or ultimately from the water), (3) the intermediate product is not a waste product. In terms of standard free energy, 65,900 calories are required at a pH of about 5, increasing as the sap becomes more alkaline, to a value of 78,500. The free energies would differ relatively little from the standard free energies (approximately 10 per cent). Such a reaction will be referred to later in discussing a paper by Linhart.

There are several organic acids whose heats of formation are greater than their heats of combustion, even greater than in the case of HCOOH. These acids are often the end and therefore waste products of anaerobic metabolism, not to say of aerobic metabolism. Indeed, Stoklasa[3] reports finding formic, acetic, butyric, and lactic acids in pure cultures of *Azotobacter*, an aerobic form. In these same aerobic cultures hydrogen gas was evolved.

Surprising as it may perhaps seem, an additional source of hydrogen

[3] Stoklasa, J., *Centr. Bakt., 2. Abt.*, 1908, xxi, 506.

gas is the atmosphere. According to Spoehr,[4] the atmosphere contains .01 per cent by volume of hydrogen. That this estimate is meritorious, and in addition one which varies little, is indicated by a summary of the experimental determinations given by Mellor.[5] The estimates of various investigators range from .019 per cent (Gautier) to .003 per cent (Rayleigh), the majority falling near the mean of these two values. In the following discussion the value of .01 per cent will be used. This particular value can hardly mislead since the probable variation from it has been indicated.

This concentration of hydrogen, instead of being small, is about equal in volume to the carbon as carbon dioxide in the atmosphere. By weight the ratio of hydrogen to carbon is 1 to 6. The corresponding ratio by weight in either proteins, carbohydrates, or fats is from 1 to 6 to 1 to 8. In bacteria[6] it is about 1 to 8. The quantities by weight of carbon and hydrogen in the atmosphere can therefore be considered equal when judged by the needs of the plant.

The following calculations will call attention, with the great suggestiveness of free energy calculations, to the potential importance of atmospheric hydrogen for plant metabolism, and in particular, for nitrogen fixation.

The equilibrium concentration of ammonia which might form from the nitrogen and hydrogen of the atmosphere, with the assistance of catalysts, is .2 ppm. in alkaline sap.

$$K = -6340/-1364.9 = 4.65$$

$$\log K = \log (NH_4OH(aq.)/(N_2)^{\frac{1}{2}}(H_2)^{\frac{3}{2}}(H_2O))$$

$$\text{activity } NH_4OH(aq.) = K \times (.8)^{\frac{1}{2}}(.0001)^{\frac{3}{2}} = 4 \times 10^{-6} \text{ M} = .2 \text{ ppm.}$$

This value of .2 ppm. corresponds to the equilibrium concentration of .1 M for nitric acid. When hydrogen is also used to form nitric acid according to the equation

$$1/2\ N_2(g.) + 5/2\ H_2(g.) + 3/2\ O_2(g.) = HNO_3(aq.)$$

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[4] Spoehr, H. A., Photosynthesis, American Chemical Society Monograph Series, New York, 1926, 36.

[5] Mellor, J. W., Comprehensive treatise on inorganic and theoretical chemistry, London, 1922, i.

[6] Russell, E. J., The microorganisms of the soil, London, 1923, 39.

the equilibrium concentration of nitrate is .16 M or 10,100 ppm., an increase of 60 per cent.

The equilibrium concentration of bases will be raised by a greater hydrogen ion concentration while that of acids will be raised by a lesser hydrogen ion concentration. Thus in the equation

$$1/2\ N_2(g.) + 3/2\ H_2(g.) + H^+ = NH_4^+;\ \Delta F^\circ = -18{,}930$$

if the sap is assumed to have a pH of about 5, the equilibrium concentration is raised to .6 ppm., a threefold increase over that in alkaline sap.

In passing, it will reward us to suggest how this large amount of hydrogen in normal air might play a rôle in the carbon dioxide assimilation of aerobic forms. For instance, if the hypothetical assimilation reaction

$$CO_2(g.) + H_2O(l) = HCHO(l) + O_2;\ \Delta F^\circ = 122{,}000$$

were to proceed

$$CO_2(g.) + H_2(g.) = HCHO(l) + 1/2\ O_2;\ \Delta F^\circ = 66{,}000$$

the standard free energy required for the fixation of carbon dioxide would be halved. In this case it is important to determine the actual free energies, since the activity of the hydrogen gas is but 1/10,000 that of water. At a concentration of HCHO of 1 part per million, calculations show that there exists a negligible difference (2 per cent and 5 per cent) between the free energies and the standard free energies. For two reasons this might have been anticipated; the standard free energy values are very large; with the exception of oxygen all the substances occur to their first powers in the activity coefficient.

While we need hardly resort to the last named equation to explain carbon dioxide assimilation by higher green plants, in view of the overwhelming evidence to the contrary which has accumulated since the time of Joseph Priestley, it may well be that those organisms, such as the sulfur bacteria, denitrifiers, hydrogen fixers,[7] elementary carbon fixers,[8] and others, which reduce inorganic forms of carbon

[7] Kaserer, H., *Centr. Bakt., 2. Abt.*, 1906, xvi, 681.

[8] Potter, M. C., *Proc. Roy. Soc. London, Series B*, 1908, lxxx, 239.

without the aid of radiant energy, derive their energy not solely by the oxidation of non-carbonaceous substances with oxygen gas, but partially, at least, by the reduction of CO_2 or other substances with hydrogen gas. In the same speculative vein it may be suggested that there is some degree of identity between those organisms which can fix nitrogen and those organisms which can utilize hydrogen.

It has now been shown that nitrogen can be fixed both exothermically and with the yielding of free energy either as ammonia or nitrate in aerobic processes and at least as ammonia in anaerobic processes.

Some may think that in nitrogen-fixing organisms the simple compounds like ammonia, nitrate, and others are never produced directly, and that what occurs is always the immediate formation of higher compounds with carbon. In other words, the plant may never be able to take proper advantage of the energy yielded by simple fixation. If so, and if energy can be plainly shown to be necessary for the arrangement of nitrogen into higher compounds, then there would be great reason to accept the view now held so widely, that energy is essential for the fixation of nitrogen.

To this argument which on the surface appears to carry weight, there are several objections. The author feels it advisable to enter rather fully into these.

First, physiological chemistry supplies no evidence which makes it probable or improbable.

Second, the formation of complex compounds from the simpler takes place very generally with the liberation of energy.

Third, to suppose that complex nitrogen compounds like amino acids can be formed in no more than one step would be contrary to the present principles of organic chemistry. To say the least, a very high (if not unheard of) order of reaction would need to be supposed to bring the direct formation of these compounds in keeping with our present information.

Fourth, leaving aside the question of an energy requirement in the formation of higher compounds from nitrogen gas, it is nearly certain that energy is not essential for their formation from ammonia. Appearance in this instance must not pass for fact. The energy needed to form the higher compounds may be the sum of endothermic changes of the carbon molecules and exothermic changes of the nitro-

gen molecules, to use a distinction not permitted at present in talking of organic compounds. If this should turn out to be true, then to say that the average of nitrogen changes requires energy would be like saying that after an assisting engine had been fastened to the rear of a freight train and was actually aiding its motion, the freight train required power to pull the engine along. It is subsequent changes of the carbon molecule rather than of the nitrogen molecule that should involve considerable changes in energy levels, owing to there being a greater range of valence change.

Fifth, an analogy to the energy change in the formation of higher nitrogen compounds may be found in the energy change when a simple non-nitrogenous carbon compound of high energy content condenses to form a carbohydrate; and without question the average free energy change of this sort is small, and of a negative character. We have the prominent instance of 6 molecules of HCHO polymerizing to form 1 of glucose. The heat of formation of HCHO according to

$$C(\text{diam.}) + H_2(\text{g.}) + 1/2\ O_2(\text{g.}) = HCHO(\text{g.})$$

has been determined only quite recently by von Wartenberg and Lerner-Steinberg[9] to be $-27{,}800$. The heat of formation of glucose is $-302{,}000$. From this we see that about 150,000 calories are liberated during this polymerization, an enormous amount which approximates one-half the heat of formation and one-quarter the heat of combustion of glucose. The conclusions are not different when free energies are considered in place of heats of reaction. Certainly the standard free energy of HCHO would be only a trifle different from the heat of formation of HCHO, but let us assume that these are equal. The value of the standard free energy of glucose has been determined from unpublished calculations by the author to be $-219{,}700$. The calculations were made by the entropy method with the use of the very excellent specific heat data containing thirty experimental points on a temperature scale which ranged from 19.1° to 287.2° absolute, obtained by Simon.[10] Consequently in the polymerization reaction, ΔF° is about $-72{,}000$. In the plant the substances

[9] von Wartenberg, H., and Lerner-Steinberg, *Z. Ang. Chem.*, 1925, xxxviii, 591.
[10] Simon, F., *Ann. Phys.*, 1922, lxviii, 241.

would hardly be in their standard states, but this value gives a good idea of the magnitude of ΔF when the reaction takes place in somewhat concentrated solutions. However, since there are six reactants to one resultant, the concentration of HCHO need not be very dilute before ΔF would become positive, in which case, true enough, energy would be needed for polymerization. In every instance of polymerization or condensation the effect of dilution is the same; that is, the greater the dilution the more positive does ΔF become. But the plant can very simply overcome this by removing the end-products so promptly that ΔF would be negative, and presumably this occurs. Thermodynamics cannot testify that the organism does this; but the very fact that the greater number of reactions in plants go merely with the aid of non-energy-supplying enzymes or catalysts, strongly supports the view that the plant must carry on its reactions at concentrations where free energy would not be required.

Sixth, the standard free energy of formation of a higher compound of nitrogen, carbon, hydrogen, and oxygen is known. For urea, ΔF° is $-47{,}280$. Thus a great deal of free energy is yielded in the formation of this higher compound. When urea is formed, not from its elements, but from two simple compounds, as in the equation

$$CO_2(aq.) + 2\ NH_3(aq.) = \text{urea}(aq.) + H_2O(l)$$

ΔF° is 5030, indicating that at standard conditions free energy is required. However, so long as urea is removed rapidly enough, then no matter how dilute the concentration of the reactants, the reaction will require no free energy. This particular reaction is typical of the reactions which ammonia may undergo with another compound to lose water, and shows that probably the standard free energy change in such condensations is small. Yet even assuming that this reaction when it occurs in a plant is at such a concentration as to require free energy, the free energy required in that event might well be drawn from a supply incidentally provided during the fixation of nitrogen into ammonia, and so the total free energy expended still remain negative.

Returning to the thermodynamic argument, there are other reactions for the free energy yielding fixation of nitrogen. HCNO (aq.) has a standard free energy of $-29{,}000$; urea, $-47{,}280$; and HNO_2 (aq.),

−13,070. These compounds may spontaneously form on condition that hydrogen gas is a reactant. Were the hydrogen, and therefore part of the oxygen, derived from water, as in the case of nitric acid discussed originally, then nitrous acid would be the only compound not requiring free energy for its formation, and this would be the case only at quite high dilution. In the cases of C_2N_2(g.), CNI(s.), HCN(aq.), NO(g.), NOCl(g.), NO_2(g.), N_2O_4(g.), and N_2O(g.), the standard free energies are positive. Accordingly, energy would be required if nitrogen is fixed as hydrocyanic acid, or as any of these substances. Since for assimilation, however, these fixed forms would be changed by reacting with water, to either an ammonia form or a nitrate form, the net result would be a yielding of free energy. For instance, in the reaction

$$HCN(aq.) + H_2O + 1/2\ O_2(g.) = NH_3(aq.) + CO_2(aq.)$$

ΔF° is −73,510. The standard free energy of formation of HCN is 27,520, consequently the net result of fixation is −45,990.

It would be profitable to consider a fixation reaction which has been the source of some confusion. The reaction for the hydrolysis of nitrogen by water,

$$N_2(g.) + 2\ H_2O(l) = NH_4NO_2$$

has a positive standard free energy of 85,690. Lewis and Randall (1923) have calculated from this that a pressure (fugacity) of nitrogen of about 10^{51} atmospheres would be required for ammonium nitrate to be formed at an equilibrium concentration of 10^{-6} M. G. Oddo[11] had measured (1915) the ionization of water in air. He found it surprisingly large and concluded that a considerable amount of NH_4NO_2 should be formed by the hydrolysis of nitrogen. Calculations show that he confused mechanism with equilibrium. Even were the water completely ionized, and $2\ H^+ + 2\ OH^-$ substituted for $2\ H_2O$ in the above equation, the pressure of nitrogen required would still be 10^{23} atmospheres. Linhart,[12] who was among the first to apply free energy data to biological problems, attempted to determine the efficiency of nitrogen fixation by *Azotobacter*. From analogy

[11] Oddo, G., *Gazz. chim. ital.*, 1915, xlv, I, 395.

[12] Linhart, G. A., *J. Gen. Physiol.*, 1919–20, ii, 247.

with somewhat related compounds, he made an elaborate calculation of the standard free energy of mannite, which was at that time unknown, even approximately. In seemingly arbitrary fashion he used the above highly endothermic and free energy demanding hydrolysis reaction (except that NH_4OH (aq.) was the resultant). While the free energy data of nitric acid and ammonia may not have been available at that time, the heats of reactions as approximations might have been considered. Of all the possible fixation reactions he may have correctly chosen the one employed by *Azotobacter*. This hydrolysis reaction possesses a large positive free energy because oxygen gas is not among the reactants. Falk and McGee[13] have obtained evidence that this hydrolysis reaction takes place to some extent in the presence of metallic iron, which they say they believe supplies the energy. Their evidence is not very extensive. The reaction goes appreciably in the electric arc, since only a few volts are needed.

There is experimental confirmation of the contentions in this paper. Workers on the nitrogen-fixing bacteria have themselves hesitated to deny that the wide ratio (of at least 1 to 50) of the nitrogen fixed to carbohydrate available might be narrowed were it possible to provide the organisms with more favorable conditions of environment. While experiments in general have not suggested that this ratio may be narrowed, Truffaut and Bezssonov[14] now present evidence that in the presence of nitrogen-fixing bacteria corn develops normally and reaches maturity in mediums devoid of organic nitrogenous matter, and that the ratio of the carbonaceous material excreted by the roots to the nitrogen fixed approaches 1 to 1. Some of the carbonaceous material would certainly serve to supply the carbon requirements of the organisms, in this way reducing the amount available for the supposed energy need in nitrogen fixation. And so, it is not at all obvious that the carbonaceous excretion functions as a source of energy. Since the carbonaceous excretion would contain about 40 per cent carbon, the ratio of excreted carbon to nitrogen fixed is only narrower. It is to be noted that in these experiments, in which according to the authors the fixation was probably done by anaero-

[13] Falk, K. G., and McGee, R. H., *Chem. and Metal. Eng.*, 1923, xxix, 224.
[14] Truffaut, G., and Bezssonov, N., *Sc. Sol*, 1925, iv, 3–53.

bic organisms (*i.e. Clostridium*), no sugar or carbohydrate excretions could be detected, but only organic acids (*i.e.* malic). The organic acids are precisely the substances some of which yield hydrogen with the liberation of free energy, as would be required were the equations presented in this paper to be employed.

There is strong evidence[15] that *Azotobacter* fixes nitrogen as ammonia.

Still more relevant is the recent extensive evidence of Christensen-Weniger.[16] He finds that the process of fixation by the nodule bacteria of several species of legumes is exothermic or nearly so. The energy requirements of the nodule bacteria were not met with by increased assimilating powers in nitrogen-fertilized legumes. Whether some of the nitrogen was supplied as fertilizer or some by fixation, the final dry weight was almost the same and from the small excess of growth in the fertilized plants he was able to fix the upper limits of the energy supply. He found the eventual requirements for the nodule bacteria so small as to be unimportant. He was quite aware of the exothermic heat of formation of ammonia.

If the nitrogen were fixed by the bacteria as nitrate, which might then be reduced in others parts of the host plant by means of radiant energy, this might in a measure explain the seemingly less efficient assimilation of nitrogen by the nodule organisms in pure culture, since there they would need more energy to reduce the nitrate.

SUMMARY.

Fixation of nitrogen even with liberation of energy or free energy, will take place if either oxygen gas or hydrogen gas, or other substances, especially gases, whose standard free energies are close to zero, are involved to form either nitrates, ammonia, or cyanide, not to speak of still other compounds. It has been pointed out that there are two and only two general conditions where nitrogen fixation can require energy. These are, first, if nitrogen reacts with some compound like water with an already high negative free energy of formation and where negligible oxidation of nitrogen would occur; second,

[15] Kostyschew, S., Ryskaltschuk, A., and Schwezowa, O., *Z. physiol. Chem.*, 1926, cliv, 1.

[16] Christensen-Weniger, F., *Centr. Bakt., 2. Abt.*, 1923, lviii, 41; *Chem. Abstr.*, 1925, xix, 2509.

if the plant does not take advantage of working at concentrations where the process would yield free energy.

If nitrogen fixation is exothermic and free energy-yielding, how is the carbohydrate requirement of nitrogen-fixing organisms to be interpreted? Are the experimental determinations of the carbon to nitrogen ratio purely circumstantial? Is further hope given to those who may experimentally try to narrow this ratio to where the carbon used is only for the carbon requirements of general metabolism, exclusive of fixation? Do not hypotheses concerning the fixation of nitrogen in the evolutionary process, which are based on the conception that energy is required, lose some of their significance? Does it not suggest that perhaps fixation is far more universal than is supposed among living forms, particularly among the higher green plants, and thereby give encouragement to those who may wish to demonstrate this experimentally? Does it not indicate that perhaps the function of fixation is often to obtain energy for use in general metabolism? Is the general carbohydrate metabolism of the fixation forms to be regarded as being merely extremely inefficient? Or most suggestive of all, is the carbohydrate serving some unobserved function?

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STUDIES ON PERMEABILITY OF MEMBRANES.

I. Introduction and the Diffusion of Ions across the Dried Collodion Membrane.

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INTRODUCTION.

When the different investigations on membrane potentials are taken into consideration, it seems that the theory of these potentials may be treated in three different ways. We may distinguish (*a*) phase boundary potentials, (*b*) mixed electrode potentials, (*c*) diffusion potentials.

The first theory assumes that the potential difference of each side of the membrane against the adjacent solution consists of a jump (a sudden, almost discontinuous fall or rise) of the potential at the boundary of two phases, the liquid being the one phase and the substance of the membrane acting as solvent for electrolytes being the other. The differences in the tendency of distribution of each single kind of ion between the two solvents which are in conflict with electroneutrality, produce the potential differences. This theory, first developed briefly by Nernst(1) and thereafter thoroughly elaborated theoretically and experimentally by Haber(2), has been applied to biological membranes by Beutner(3). The latter author also found different models in which he assumes the same mechanism, and at the same time, assumes that no essential part of the P.D. observed is produced within the membrane itself. He could reproduce membranes the potential of which depends on the concentration of any cation dissolved in the adjacent solution, and does not depend only on the concentration of one particular ion, as is the case with metallic electrodes and with the glass membrane, at least in certain kinds of glass such as Haber and

Klemensiewicz(4) used in their investigations. Beutner's assumptions meet the following difficulties. In general, it can be shown that a membrane consisting of a water-immiscible phase and behaving as a solvent for the dissolved substances, cannot give any appreciable "conconcentration effect," that means: any appreciable P.D. when placed between two solutions of one electrolyte in different concentrations(5). In order to explain that such effects are brought about none the less, Beutner has to assume that in the oily phase there is a small amount of a weak acid (*e.g.* a trace of salicylic acid in salicylicaldehyde), which is scarcely soluble in water, and that such a weak acid dissociates in the oil very strongly, like a strong acid or a salt, so that the reaction (written in terms of salts, not of ions)

$$\text{salicylic acid} + KCl \rightleftarrows K \text{ salicylate} + HCl$$

will proceed amply in the direction $\rightarrow$, while it is known that this reaction in aqueous solutions practically goes on only in the direction $\leftarrow$. This assumption is necessary for Beutner's theory, but the experience of the last decade does not at all confirm such a behavior of weak acids in oils.

The second theory may be applied in such cases where the membrane has the character of an electrolyte-like substance (*e.g.* silicates, such as glass, permutit). One of the ions of the membrane substance may be an inert or "colloidal" ion such as the silicate ion, while the other ion which happens to be combined with the silicate (*e.g.* Na) shows what may be called an electrolytic tension towards the solution, like a metallic electrode. This ion, which forms a component of the solid silicate, may be partially exchanged for another ion present in the solution (*e.g.* H^+). Then the silicate behaves like a mixed Na and H electrode. The potential difference will depend on the kind and concentration of any such ions which are combined or are able to combine with the silicate in exchange for the ion originally combined with the silicate ion. Recently Horovitz (6–8) showed that this mechanism holds for the membrane potential produced with certain kinds of glass.

A third theory attributes the potential differences produced by membranes to the difference in the velocity of the single ions diffusing across the membrane. According to this theory, there is no abrupt

change in the potential at the boundary of the membrane but a gradual change of the potential from the one boundary towards the other, as is the case with a liquid junction potential when two different electrolyte solutions are in direct contact with each other, and by diffusion or convection a zone of gradual transition between the two solutions is interposed. So, this third theory is that of a diffusion potential. In a previous paper(9) the theory of such membranes as dried collodion or apple skin was developed on this basis.

It does not follow that these three theories are contradictory to each other. It may happen that two or even all three of these possibilities are combined in the same membrane. For instance, Beutner will probably not exclude the possibility of a diffusion potential within the membrane when it is in contact with two different solutions at the two sides. He only assumes that this diffusion potential plays quantitatively a negligible part in the total observed potential difference. On the other hand, Baur(10) endeavored to prove that the diffusion potential plays the predominating part and the abrupt change of the phase boundary potential may be neglected. Cremer(11), who was perhaps the first to emphasize the importance of these considerations, left it undecided which of these sources of potential differences plays the important rôle, emphasizing rather more the diffusion potential. The difficulty of the theory of diffusion potentials seemed to be that under any known condition the diffusion potentials, except the ones produced by acids of alkalis, are not great enough to give such a great effect as the membranes sometimes do. The differences in the mobilities of the different kinds of ions, except H^+ and OH^- ions, are not sufficient. We shall show, however, that in certain membranes these differences are enormously greater than the well known small differences in aqueous solutions. It was the aim of the author (9,12) to show that for a membrane such as collodion(12,*d*) the theory of diffusion potentials is sufficient to explain all known facts. The theory of mixed electrodes need not be considered because the chemical nature of collodion (and of some lipoid membranes, such as the wax in apple skin) excludes this possibility. The substance of this membrane is not an electrolyte-like material nor does it consist of a cation and anion like a glass. However, the question may be asked whether the phase boundary theory may not be applied for collodion.

Now, for the usual permeable collodion membrane with its large pores, obviously the whole membrane effect is due only to these pores. There can be no doubt that in any of the well known dialysis experiments the dialyzing salts will go through the pores of the membrane and will not go through the substance of the collodion itself to any appreciable extent. Again, the transition from the usual large pored collodion membrane to the dried membrane is quite gradual. So it seemed most probable that the effect of the dried collodion membrane is due to the same mechanism as that of the usual collodion membrane. The dried membrane is to be regarded only as a practically obtainable limiting type of porous membrane with decreasing pore sizes.

Since the so called Donnan membrane potential has played a great part in researches on membranes in the last decade, it seems necessary to explain the relation of the Donnan potential to the different kinds of potential mentioned above. The difference is the following. All of the three mentioned theories attempt to trace the course of the potential from the one side of the membrane to the other. Two of the theories assume a more or less abrupt change of the potential at each boundary but no change elsewhere. The other theory assumes a gradual transition of the potential from the one boundary to the other. The total potential difference is made up by the sum of the single abrupt changes, or the integral of the single differential changes, according to the assumption. The Donnan theory is not concerned at all with the course of the potential across the membrane; it only takes into consideration the difference between the two sides of the membrane, and it can be applied to any case where one kind of ion, which is present only on the one side of the membrane, is permanently not present on the other side. Donnan stated as the necessary condition for the possibility of such a case occurring the non-diffusibility of one kind of ion across the membrane, *i.e.* the lack of any mobility across the membrane. However, the same condition may be the limiting case either for very low mobility of this particular ion in the membrane, or for a very low solubility of this ion in the membrane. In either case the liquid on the other side of the membrane remains permanently free from this particular kind of ion and the conditions of the Donnan equilibrium are fulfilled. In such a case, where either the mobility or the solubility of an ion within the membrane is really

zero, there is an abrupt change of the potential at the boundary of the membrane. However, the Donnan theory does not consider the course of the potential at all, and it deals only with the difference of the potential between the two sides. Neither does the Donnan theory concern itself with the mechanism of the impermeability of this particular ion. It holds at least for the limiting cases of two quite different mechanisms: insolubility and immobility. In reality, however, not only do the limiting cases occur, but also cases where either the solubility or the mobility of the ion in the membrane is only diminished and not completely abolished. Thus our system includes the Donnan potential as a limiting case among different possibilities.

It is also possible to develop the theory of the porous membrane upon the assumption of *different phases*. Certainly, the solubility, the activity, the osmotic pressure of any dissolved substance, and the vapor pressure of the solvent, are different in the bulk of the solution from those obtaining within the capillary pores and channels. One could speak, therefore, of a coefficient of distribution or partition, between the bulk and the capillary space, of any particular substance which is a common component of both. However, such an attempt would lead to great difficulties and is of no advantage whatever. The capillary spaces should not be considered as phases separated by a sharp boundary from the bulk phase, but one should rather conceive the capillary spaces as being analogous to the surface layer of any phase in contradistinction to the bulk. Thus the entire surface of all the capillaries of a sieve-like membrane is an enormously enlarged surface of the solution. We emphasize this idea in order to avoid a discussion as to whether the theory of phase boundary potentials ought to be applied to sieve-like membranes.

The problem of the potential differences produced by membranes between two solutions is closely connected with the problem of the permeability of the membrane. In the various attempts to apply the different theories to the selective permeability of the membranes of living cells, the conception of a lipoid membrane as a solvent had been chiefly used, because of the influence of the well known studies of Overton. Here the membrane was assumed to be a homogeneous phase with sharply defined boundaries interposed between the inner phase (the protoplasm) and the solution in contact with the exterior

of the cell. But since this theory was not satisfactory for every case, the idea of a mosaic membrane has been suggested by Nathansohn (13). This is supposed to be a porous membrane, a sieve consisting of a framework of a lipoid the pores of which are filled by an aqueous solution. Here we have the sieve-like membrane which only differs from the collodion membrane insofar as the substance of the framework is not quite inert but participates in the permeability for certain substances which are soluble in it. In any case, in order to separate these two alleged effects of the mosaic membrane we must first study the effect of a sieve membrane with an inactive framework in which the whole problem of permeability depends on the pores. For that purpose the dried collodion membrane seems to be the almost ideal model, because no other porous membrane of sufficient mechanical resistance (clay, etc.) can be obtained so easily with pores as small as in collodion. M. Traube's copper ferrocyanide membrane suffers from the lack of mechanical strength and requires always the presence of the membrane-forming substances, $CuSO_4$, on the one side and Na ferrocyanide on the other. This complication makes it almost impossible to study the properties of such membranes with those methods which proved the most suitable and simplest in the case of collodion membrane. Collander(14) has recently made a very exhaustive study of the copper ferrocyanide membrane.

1. The Problem of Direct Diffusion Experiments.

In a series of experimental studies on the dried collodion membrane (12, *d*, *g*, *h*, *i*) it has been shown that this membrane is considerably less permeable for anions than for cations. A first attempt at a theoretical treatment of the properties of such a membrane has been made in a previous paper in this journal (9).

The assumption of a relatively small permeability for anions was founded at the outset of these studies upon the following interpretation of the E.M.F. of concentration chains. When two solutions of one electrolyte in different concentrations are separated by a dried collodion membrane, a potential difference is established which in the best cases reaches the theoretical maximum value of an ordinary concentration chain with electrodes reversible for the cations. The simplest inter-

pretation of this effect was the assumption that the anions are not, or at least are much less, mobile in the pores of the membrane than the cations. Such an assumption must, of course, be proved by direct diffusion experiments. These diffusion experiments are the subject of this paper.

In some of the previous papers (12, *d*, *i*) diffusion experiments have already been described which fairly well confirmed the assumption, but these experiments have not been perfectly satisfactory. The difficulties consisted in the following circumstances. In the first place, when the membrane was made thick enough to resist the mechanical strain in such an experiment, the time required to yield quantities of the diffused ions sufficient for chemical analysis was very long, weeks, even months. Very often the membrane did not retain its original properties for such a long time, and many experiments were spoiled in this way. On the other hand, when the membrane was made thin enough to allow a sufficient diffusion in 1 or a few days, the lack of mechanical resistance spoiled many experiments. The membrane became leaky and the leaks could be shown sometimes simply in the ordinary macroscopic way; sometimes the leaks were relatively small so that they were not manifested macroscopically, but produced a potential difference between two KCl solutions, 0.1 and 0.01 N, much smaller than the expected maximum value. This value is, theoretically, as has been shown (9), 55 millivolts, and good membranes, give, in fact, a potential difference of 50 to 53 millivolts. Leaks not large enough to be visible macroscopically become manifest by a drop of this potental difference sometimes down to 25 millivolts or much less. Nevertheless, selecting the good experiments, it could be shown, that HCl diffuses against pure water across a membrane extremely slowly, while HCl and KCl exchange cations relatively quickly across the membrane. Other arrangements for diffusion experiments gave similar results. But all of the results so far obtained are only qualitative and not really satisfactory. In order to obtain reliable results a kind of membrane had to be employed which was permeable enough to give a measurable amount of diffusion in a few days, and which, on the other hand, retained its great difference of behavior towards cations and anions, and which also retained its properties during the time of the experiment. The authors finally

succeeded in obtaining the required kind of membrane by selection of a suitable kind of collodion and method of making the membrane.

2. The Method of Preparing and Standardizing the Membrane.

The general method of preparing this kind of membrane consists in pouring some solution of collodion into a cylindrical glass vessel, letting a film of collodion form adhering to the glass wall, permitting it to dry to a certain degree, and then, in distinction to the way in which the ordinary collodion membrane is made, not to detach it from the wall by means of wetting it with water, but simply by means of forceps or the fingers. The drying has to proceed rather far to allow this detachment. The best way is to wait until the detachment begins spontaneously. After the membrane has been pulled out of the glass vessel it must be dried further, for at least a day, in the open air. Several variations in this method are possible and we shall describe the one which seemed the most convenient.

When different kinds of collodion, even when dissolved in the same solvent medium, are employed, the properties of the membranes may vary to a great extent. The different samples of membranes made up from the same collodion solutions behave, though not completely, relatively fairly uniformly. As the best method of characterizing such a membrane the measurement of the potential difference between an 0.1 and an 0.01 M KCl solution separated by the membrane may be recommended. This may be called the *concentration potential* or the *Co P* of the membrane (12, *i*). This *Co P* depends to a considerable extent on the kind of collodion used. At least ten different samples of collodion were used, such as "parlodion," "commercial gun cotton No. 1," and several samples of nitrocellulose of different nitrogen content. None of these gave really good results. Some of them gave at times membranes with a relatively high *Co P*, such as "gun cotton," but were so poorly permeable (which could also be shown by the very poor electric conductivity in an aqueous solution of some neutral salt) that they were useless for diffusion experiments. Others had a better permeability but had such a low *Co P* (between 25 and 40 millivolts) that the required specific properties of the membranes were not obtained. Such membranes showed, in

fact, only relatively small differences in permeability for anions and cations. For instance, an 0.1 N solution of HCl did not diffuse into pure H_2O across such membranes much slower than it did into a KCl solution. The one sort of collodion which was found satisfactory for these purposes was "Celloidin-Schering." Membranes may be made up either in a flat form, which will be more fully discussed in a subsequent paper, or in the form of bags. For diffusion experiments the bag form seemed to be preferable on account of the greater surface, though for many other purposes the flat membrane is preferable. The following method turned out to be most suitable for the required purposes.

5 gm. of commercial celloidin shreds, Schering, previously dried in the air or by short washing with absolute alcohol, are dissolved in a mixture consisting of 75 cc. of absolute alcohol and 25 cc. of anhydrous ether. The process of solution may require several days and is accelerated by frequent gentle shaking.

Suitable tubes, such as 50 cc. round bottom centrifuge tubes, are filled with the above collodion solution, permitted to stand covered until the air bubbles rise to the top and disappear, after which the greater portion of the collodion is poured off leaving about 5 cc. in the tube. This residual amount is then carefully distributed in as uniform a layer as possible by slow rotation and warming in the palms of the hands. After several minutes of such rotation and evaporation of the solvents, the tube is placed in a clamp in an inverted position, mouth downward, and permitted to drain. As stated above, it is best to leave the tube undisturbed until the membrane begins to detach spontaneously from the glass. This may require under various atmospheric conditions 2 to 4 hours. The rim of the membrane is now cut with the point of a knife and it is removed by careful and gentle pulling. Special care must be exercised not to exert too great a strain upon the bottom of the membrane, so as not to stretch it too much at that place. At this point the membrane is still quite elastic, and the distortion due to the manipulation of detachment from the glass may be corrected by gently blowing into it several times during the next 5 to 10 minutes. The bags are now placed upon a clean surface and allowed to dry in free air for about 1 or 2 days.

It will be observed that at the end of this drying period all mem-

branes show a greater or lesser degree of shrinking and wrinkling. The wrinkling can be partially prevented by repeated blowing during the process of the drying. The degree of this shrinking appears to depend upon the extent of drying previous to the removal of the bag from the glass tube, and upon the thickness of the collodion layer. As a rule longer drying within the glass tube and the thinner layers yield the smoother membranes. It may be noted, however, that a moderate degree of shrinking does not impair a membrane for the purposes described. A membrane which is entirely smooth is as a rule not very resistant to mechanical strain. On the other hand, strongly shrunk and wrinkled membranes may be unserviceable because of lack of uniformity of shape and texture. But the characteristic properties of permeability, conductivity, in a given electrolyte solution, and of the concentration potential seem to depend to only a small extent upon the differences in the amount of shrinking and on the relative shape. It is true that the degree of shrinking is not entirely without effect. Recently Liesegang (15) reported that collodion membranes, when prevented from shrinking during the process of drying, are much more permeable than the shrunk ones. This fact has been known to the authors for a long time, though it has not been expressly published, since the interest was directed towards obtaining the limiting case of membranes with the narrowest pores possible. It has also been known to the authors, *e.g.* that a collodion membrane made by impregnating a filter paper bag (extraction shells of Schleicher and Schüll, or simply filter paper) is much more permeable than the membrane without a skeleton, even when completely dried. The *Co P* of such membranes never exceeded about 40 millivolts. For that reason this kind of membrane was abandoned by the authors, though it may be useful for other purposes.

A completely dried membrane is a perfect electric insulator and is highly electrified by gently rubbing it against the hair. Not being easily wetted by water, it retains its electric charge a very long time, even in a humid atmosphere. This property of an electric insulator may be emphasized because it shows that the subsequently described properties of permeability and electric conductivity are due to the pores and their contents and not to the chemical or physical nature of the solid nitrocellulose substance.

A quantitative expression of the most important characteristic property of these membranes is obtained by measuring the concentration potential (*Co P*) between an 0.1 N and an 0.01 N KCl solution separated by the membrane. A suitable arrangement for this measurement is shown in Fig. 1. The advantage of KCl solutions is chiefly in the lack of any diffusion potentials against the solutions of the calomel electrodes. As was stated above the best membranes will show a *Co P* at room temperature of 50 to 55 millivolts. This

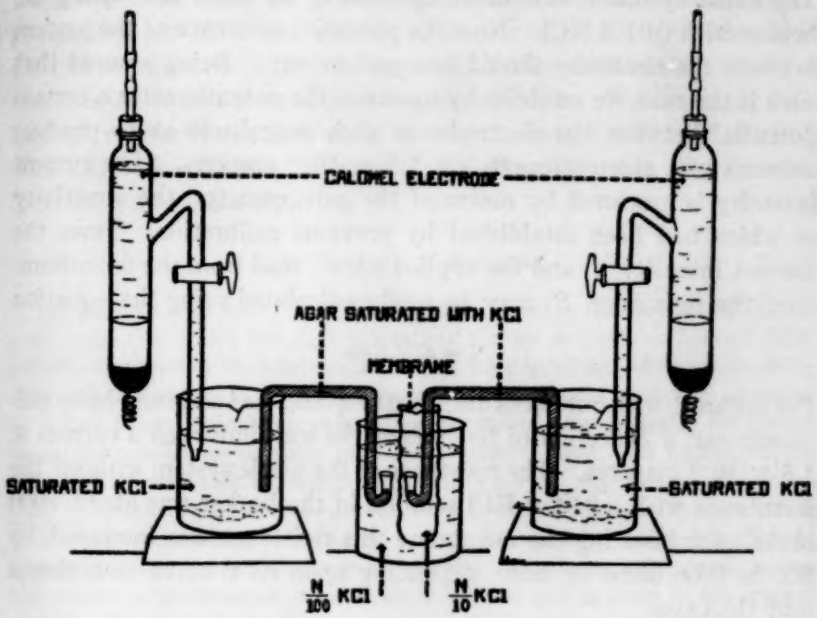

FIG. 1.

Co P may be reproduced on the same day for any membrane within a fraction of a millivolt, and within a longer period of several days or even weeks, the *Co P* of the same membrane will not vary as a rule more than 3 or 4 millivolts. The best way of preserving the membranes is to immerse them in and fill them with distilled water.

Although the electric conductance of the membrane immersed in a solution of electrolytes does not come strictly within the scope of this paper, we cannot avoid touching upon this subject. The

resistance of such a system is different, of course, in the direct and alternating currents for well known reasons. The resistance which interests us from the standpoint of the permeability of the membrane for ions is the ohmic resistance to a direct current, since the magnitude of the conductance in a given electrolyte solution is in the sense of Nernst's theory a certain function of the diffusibility of the ions.

The conductance of a membrane may be approximately measured in the following simple manner using the arrangement shown in Fig. 1. The entire system is first made up *omitting the membrane*, filling the beaker with 0.01 N KCl. Now the potential difference of the system between the electrodes should be equal to zero. Being assured that such is the case, we establish by means of the potentiometer a certain potential between the electrodes of such magnitude as to produce currents of a given strength, *e.g.* 1.5×10^{-7} amperes. The current intensity is measured by means of the galvanometer, the sensitivity of which had been established by previous calibration. From the current intensity, I, and the applied E.M.F., read from the potentiometer, the resistance, R, may be easily calculated using the equation

$$I = E.M.F./R$$

For instance, with our Leeds and Northrup enclosed scale and lamp galvanometer, a deflection of ten lines on the scale indicated a current of 1.5×10^{-7} amperes. The resistance of the whole system without the membrane with a 0.01 N KCl solution in the beaker was about 3000 ohms. On inserting the membrane this resistance was increased by 600 to 8000 ohms or more, depending upon its relative smoothness and thickness.

It was surprising to find that such relatively high conductance was compatible with such highly pronounced specific influence on the relative mobilities of the cations and anions. To produce such differences in the mobilities, as are described later in this paper, we must assume extremely narrow pores. The high conductance of the membrane indicates that the number of these fine pores must be very great. Thus, for instance, for membranes prepared from a certain brand of gun cotton and which yielded about the same *Co P* effect, the resistance, under the same conditions, was found to be enormously greater, up to several million ohms, showing that in such membranes

the number of pores was correspondingly smaller, while the size of the pores upon which the *Co P* depends is probably the same. On the other hand, we have observed membranes prepared from other kinds of collodion, *e.g.* parlodion, yielding much lower *Co P* values (about 25 to 30 millivolts) with a higher resistance than in the celloidin membranes. Such membranes probably have larger but fewer pores. Hence the usefulness of our celloidin membranes is probably due to a large number of very fine pores. The size of these pores is apparently within the size order of larger single molecules, for, as shown in a previous paper (12), non-electric molecules in which hydration and electric charge do not play an appreciable rôle, diffuse through these membranes at a rate corresponding to their size. For instance, urea diffuses relatively readily, while the sugars do not diffuse at all, and with aliphatic alcohols the rate of diffusion decreases rapidly with increasing molecular size.

3. *Methods of Analysis.*

The methods used were: Potassium was determined by the method of Kramer and Tisdall (16) which was slightly modified for our purposes as follows: After suitable evaporation the whole or an aliquot portion of the solution containing about 0.3 to 0.05 mg. of K in a volume of 2 cc. was precipitated with an excess (2 cc.) of the cobalti-nitrite reagent, centrifugalized after standing for several hours, the precipitate collected on a small asbestos filter, and washed on the filter several times with a dilute (approximately 0.01 M) magnesium sulfate solution. This solution was used to prevent the colloidal dispersion of the precipitate which occurs on washing with distilled water. The asbestos mat and precipitate were quantitatively removed to a small flask and titrated with N/50 permanganate and oxalate in the presence of an excess of sulfuric acid as given in the original method. Frequent checks showed that with this procedure we could determine with an accuracy of about 2 to 5 per cent analogous quantities of K in known solutions of KCl, in the presence or absence of the other salts used in our experiments.

The chloride ion was determined by the well known Mohr method using the following procedure. Since the amounts to be determined were almost always extremely small, the solutions were cautiously evaporated to dryness and the residue dissolved in 1 or 2 cc. of a 10 per cent K_2CrO_4 solution. (The chromate solution had been previously treated with a little $AgNO_3$ solution to remove traces of chloride present, and after standing for a day or 2, filtered.) The titration was carried out with N/100 $AgNO_3$ solution using microburette with a fine tip. The end-point of the titration, the first appearance of a clear brown

tint, is best observed when the titration is performed in a white porcelain dish or in a glass vessel standing directly on a white surface. The excess of $AgNO_3$ necessary to produce this perceptible red brownish shade was found to amount, under these conditions, to about 0.02 to 0.05 cc. (a small drop) of the 0.01 N solution, which amount must be subtracted from the titration figure.

4. *The Diffusion Experiments.*

Several groups of experiments were carried out as follows:

I. 0.1 M KCl against H_2O Table I, A.
0.1 M KNO_3 " 0.1 M NaCl, Table I, B.
0.1 M KCl " 0.1 M $NaNO_3$, " I, C.

II. The same experiments were carried out, but in this case each membrane was used for repeated diffusion experiments with increasing periods of time of diffusion. The solutions outside and inside the collodion bag were renewed at the end of each period, Table II, A and B.

III. In Table III are detailed experiments using the same salts as above but in much higher concentrations, 0.5–1.0 M.

IV. Three experiments were carried out in which the membranes separated 0.1 M electrolyte solutions with the cation (K^+) in common but with different anions (Cl^- and NO_3^-), as shown in Table IV.

V. Table V shows a series of experiments in which the molar concentrations of the two electrolytes separated by the membrane were deliberately chosen in the ratio 1:5 with the chloride having the higher concentration. In this arrangement the P.D. of the Na^+ solution against a K^+ solution is almost completely abolished, as will be more fully explained in the discussion of the results.

VI. The experiments detailed in Table VI are presented to show the effect of low *Co P* values of the membranes and also to show that some kinds of collodion are not suitable for the purposes of approaching the characteristics of an ideal semipermeable membrane for ions. The membranes used in Series I to V were prepared for Celloidin-Schering, whereas the membranes used in this series were made from other commercial brands of nitrocellulose.

TABLE I.

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl	Diffused K per day (24 hrs.)
	Before	After		K	Cl		
	mv.		hrs.	mols × 10^{-4}			mols × 10^{-4}
A. Diffusion of 0.1 M KCl against H_2O							
1	53.8		24	2.0	4.8	0.4	2
		42.0	42	1.6	3.5	0.5	0.92
2	51.5		70	5.6	5.1	1.1	1.3
3	49.8	46.7	70	3.6	3.2	1.1	1.2
B. Diffusion of 0.1 M KNO_3 against 0.1 M NaCl							
1	53.0		24	9.1	1.8	5	9.1
2		37.5	42	23.8	3.2	7	13.6
3	52.0		24	20.3	2.0	10	20.3
4		49.2	42	36.4	3.0	12	20.8
5	52.0	54.3	44	91.6	1.8	51	20.7
6	47.5	54.3	44	59.6	1.5	40	11.5
7		50.2	92	11.0	1.0	11	2.9
8		20.2	120	35.0	1.0	35	7.0
9		48.5	120	16.4	1.9	9	3.3
C. Diffusion of 0.1 M KCl against 0.1 M $NaNO_3$							
1	53.0		24	3.6	0.8	5	3.6
2		53.5	42	6.1	0.6	10	3.5
3	53.5	51.2	70	13.8	1.4	10	4.7
4	53.5	50.0	70	65.5	4.9	13	22.5

In Series A, where KCl diffused against pure water, the diffused amounts of K^+ and Cl^- are extremely small and, within the experimental error, equivalent to each other.

In Series B and C, where the membrane separated two electrolyte solutions, the amount of K^+ diffused is much higher, and the ratio of diffused K^+:Cl^- is within the range of 4 to 20.

TABLE II.

Progressive Diffusion Experiments with 0.1 N Solutions.

A. KCl against $NaNO_3$

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl
	Before	After		K	Cl	
	mm.		*days*	*mols* × 10^{-8}		
1	51.5		3	1.6	<1	—
			6	3.3	<1	—
		52.2	12	7.2	1.7	6.6
2	51.0		3	4.6	<1	—
			6	10.8	1.7	6
		37.0	12	24.2	7.4	3
3	52.4		2	14.2	1.8	7.5
			4	31.0	3.2	9.7
			8	70.0	8.0	8.8
		48.8	12	94	13.2	7.0
4	53.7		2	6.6	1.2	5.5
			4	15.0	1.2	12.5
			8	33.0	2.6	12.7
		52.7	12	50.0	5.1	10.0
5	52.7		2	11.6	2.4	5.0
			4	23.4	5.2	4.5
			8	59.0	13.9	4.2
		47.4	12	96.0	16.0	6.0
B. KNO_3 against NaCl						
1	51.0		3	14.3	4.3	3.3
			6	32.2	3.2	10
		41.0	12	67.0	9.2	7
2	53.1		2	11.6	1.2	10
			4	25.0	1.5	16.6
			8	56.0	2.6	21.5
		46.5	12	85.0	5.2	16.3
3	50.2		2	28.3	5.6	5
			4	63.5	9.4	6.6
			8	150	30	5
		41.0	12	214	52	4.1

In this series each membrane was used for a number of successive experiments, with progressively increasing diffusion time. Again, the value of the ratio of diffused K:Cl approaches about 10, sometimes remaining almost the same in the successive experiments, sometimes with a tendency to an increase followed by a decrease in the ratio.

TABLE III.

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratio K:Cl
	Before	After		K	Cl	
	mv.		*hrs.*	*mols* × 10^{-6}		
A. Diffusion of 0.5 M KCl against H_2O						
1	54.6	50.8	20	1.0	0.8	1.2
2	55.0		92	21.5	18	1.2
B. Diffusion of 1.0 M KCl against H_2O						
3	50.8	50.7	48	0.95	0.85	1.1
4		46.3	216	1.13	1.62*	0.1 appr.
C. Diffusion of 0.5 M KCl against 0.5 M $NaNO_3$						
1		42.5	20	8.5	7.0	1.2
2		42.6	92	13.8	7.0	2.0
3		37.2	360	160.0	155.0	1.0
4		45.2	120	31.0	23.5	1.3
5		51.7	120	37.0	40.4	0.9
6	51.8	51.2	120	84.0	62.5	1.3
7	54.6	50.8	120	96.0	58.0	1.7

In the above experiments with 0.5 or 1.0 M solutions instead of 0.1 M the ratio of diffused K^+:Cl^- is decidedly lower, being approximately the same whether the diffusion takes place against water or another electrolyte.

* Analysis uncertain.

TABLE IV.

Diffusion of Anions Only.

Diffusion of 0.1 M KNO_3 against 0.1 M KCl

Membrane No.	*Co P* of membrane		Time of diffusion	Cl^-
	before	*after*	*days*	mM
1	51.5	49.7	12	0.0048
2	51.2	51.0	12	0.0068
3	51.2	39.6	12	0.400

Experiments 1 and 2 show the extremely slight amount of Cl ion diffused in a period as long as 12 days. Experiment 3 shows that a much larger amount of Cl ion diffuses when the character of the membrane has been impaired as shown by the relatively large drop in the *Co P* to 39.6 millivolts.

TABLE V.

Diffusion of 0.05 M KNO_3 against 0.25 M NaCl (No P.D. between the Separated Solutions).

Membrane No.	*Co P* of membrane		Time of diffusion	Diffused amounts		Ratios	
				K	Cl	K:Cl	$U_{K^+}:V_{Cl^-}$
	before	*after*	*hrs.*	*mols* $\times 10^{-6}$			
1	52.4	54.3	196	81	60	1.4	7
2	50.3	52.4	196	47	8.0	5.9	30
3		50.6	240	52.2	3.2	16	80
4	49.3	47.8	196	25	9.6	2.6	13
5	52.4	54.3	196	81	60	1.4	7
6	48.6	47.2	196	34.2	22.0	1.6	8
7		46.0	144	14.8	0.5	30	150
8	57.0	41.5	144	5.0	1.5	3.3	16
9	56.5	39.5	144	7.6	1.1	7	35
10	50.3	37.2	196	48	124.0	0.4	2
11	52.0	24.8	144	7.3	2.0	3.7	18
12	51.0	23.5	144	15.7	14.9	1.00	5

Though the concentration fall of chlorine is five times as great as that of potassium, the amount of diffused K^+ is always greater than that of Cl^-, except in experiment No. 10. The last column shows the mobility ratio of K^+ and Cl^-, referred to the same concentration gradient. This ratio is always greater than 1. The exceptionally high value in No. 7 may be due to an error in the particularly low amount of Cl^-. The experiments are arranged according to the *Co P* values after the experiments. However, the figures of the last column do not follow the same order. It should be noticed that the *Co P* of many membranes became much smaller after the experiment, showing that the character of the membranes are somewhat changed during the diffusion. This may explain the differences in the ratios.

TABLE VI.

Diffusion Experiments with "Gun Cotton" Membranes of Lower Co P 0.1 M KNO_3 against 0.1 M NaCl.

Membrane No.	*Co P* of membrane		Time	Diffused		$K^+:Cl^-$
	Before	After		K^+	Cl^-	
	mv.		*days*	*mols* $\times 10^{-6}$		
1	18.5	26.5	8	7.3	7.1	1.0
2	30.7	22.5	8	87.0	46.9	1.9
3	23.3	23.0	8	9.3	8.3	1.1
4	26.5	26.1	8	35.0	19.4	1.8
5	29.0	22.5	8	10.0	6.2	1.6
6	28.7	23.1	8	64.0	32.8	2.0
The same with "parlodion" membranes						
7	20.0	21.5	8	1.94	2.3	1
8		16.6	26	2.6	2.6	1
9		19.1	26	13.5	8.4	1.6
10		24.3	26	15.8	9.0	1.7
11		23.8	26	23.3	14.0	1.7

This table shows, that in membranes characterized by a low *Co P* (probably due to a larger pore size), the K:Cl ratio approaches 1 and that the specific effect of the membrane on the mobilities of cations and anions disappears.

Discussion of the Diffusion Experiments.

It can be seen from the above tables that the diffusion of an electrolyte (KCl in Table I, A) is slower across the membrane into pure H_2O than into another electrolyte solution. Though the membranes are not uniform enough as to thickness and other properties to permit an exact quantitative comparison of experiments with different membranes, still it can be seen from Table I, that on the average the amount of K^+ diffusing per day is much less in A than in B or C. This comparison indeed is not very reliable on account of differences in thickness and other properties of individual membranes. However, what is certain is the fact that the amount of diffusing Cl^- corresponds to the amount of K^+ in the diffusion against water (A), whereas it constitutes but a small fraction of the K^+ in the diffusion against another electrolyte (C). In Table I, A, the amounts are so extremely small, that an exact agreement of the analyses cannot be expected. The error of the methods in these very small ranges tends always to yield high values for Cl^-. In any case the order of magnitude for the K/Cl ratio is 1:1, whereas this ratio in B and C is on the average 10:1, varying from 7:1 to 50:1. Only in two experiments in which particularly small amounts had to be analysed the ratio came down to 5:1. Here the error of the method may depress the value, and even if it does not, the ratio 1:1 is far from being reached.

Special attention should be drawn to the experiments shown in Table II, A. Here every membrane was used in a series of successive diffusion experiments with the same combination of electrolytes (KCl against $NaNO_3$) lasting for progressively increasing periods. First it can be seen that Membrane 2, for example, in which the *Co P* dropped in the course of the experiments from 51 to 37.0 millivolts, gives relatively low ratios for the diffusion of K and Cl, dropping down to 3, while the membranes with a higher and more stable *Co P* give ratios of about 7 to 12. In general, within the limits of error, this ratio is approximately the same, though not quite exactly so. It seems that these irregularities are greater than the limits of error in the analyses, and that the regularity and reproducibility of these experiments is not perfect.

The same remarks hold for similar serial experiments with 0.1 M

KNO_3 against NaCl in Table II, B. In general, the amount of potassium diffusing seems to follow a straight line plotted against the time, whereas the chlorine diffusing shows decidedly less regularity. This may be partially due to the greater difficulty in the quantitative determination of these minute amounts of chlorine, but we believe this is not the sole factor.

All of the above experiments were carried out with 0.1 M solutions. In higher concentrations this effect becomes less and less pronounced. So in Table III, with 0.5 M solution of KCl, the amounts of K^+ and Cl^- diffusing are approximately equivalent to each other, both when the diffusion takes place against H_2O and against a 0.5 M solution of $NaNO_3$. It may be recalled that in such high concentrations the P.D. between two KCl solutions of different concentration vanishes (12, *i*), to which problem we will refer in a later communication.

The experiments shown in Table IV in which the cation on both sides of the membrane is the same, but the anions are different, demonstrate that the anions diffuse with extreme slowness, even though the possibility of exchange is present. Membrane 3 of this series, the only one through which a much larger amount of Cl^- diffused, shows at the same time a considerable drop in its *Co P* during the experiments, while Membranes 1 and 2 maintained their original properties as evinced by only small change in their *Co P* values.

A special discussion is required for the experiments shown in Table V. Here an 0.05 M KNO_3 solution was diffusing against five times as concentrated a solution of NaCl. Thus the gradient of the Cl^- concentration across the membrane was five times as great as that of the K^+ ions. None the less, the amount of diffused K^+ is higher than that of Cl^-. If we assume that the velocity is proportional to the driving force, the specific velocities of K^+ and Cl^- can be calculated, in relative terms, by dividing the diffused amount of Cl^- by five. Thus we obtain the ratio of mobility of Cl^- and K^+ shown in the last column of Table V. The reason for carrying out this kind of experiment was the following. In the other experiments with equal concentrations of KNO_3 and NaCl, a potential difference is established. Any solution of a K salt shows a P.D. against any solution of an Na salt in equal concentration across the membrane, which in the best membranes reaches about 50 millivolts (12, *d*). Now, the driving force

in the movement of a single species of ion through the membrane is the algebraic sum of the driving force resulting from the concentration difference of this ion on the two sides of the membrane and of the force of the electric field. It is not only the concentration fall which determines the rate of diffusion. Since the side with the Na solution is positive, there is, beside the osmotic force, an additional force, which is directed for positive ions from the Na solution towards the K solution and for negative ions in the opposite direction. Therefore, when the concentration fall for K ions from left to right is the same as the concentration fall for Cl ions from right to left, the total driving force is different for K and for Cl ions. The K ions are retarded, the Cl ions are accelerated by the electric forces. The specific mobility of an ion is its observed speed of movement divided by the driving force. But since the driving force is complicated, the calculation is difficult. The ratio of observed diffusion for K^+ and Cl^- does not represent the ratio of the specific mobilities of these ions. Now, by a suitable arrangement in the concentrations of the K and Na salts, the conditions can be made such that practically no P.D. arises between the two solutions. This is the case when the concentration of the Na salt is about five to ten times as great as that of the K salt. The exact ratio necessary to bring about the complete abolition of the P.D. depends on the individual P.D. of a K-Na chain with the particular membrane. But for a ratio of concentrations 1:5 the P.D. is in any case so low, that the electric force is negligible in comparison with the osmotic force. In fact, the P.D. in the arrangement of the experiments of this series, Table V, was measured in several cases and found never in excess of a few (0.5 to 2.0) millivolts, whereas the same membranes, when interposed between a NaCl and a KNO_3 solution in *equal* concentrations, showed a P.D. of 40 to 50 millivolts according to their individual properties and in agreement with the earlier findings with KCl-NaCl chains (12, *d*). Therefore, the driving force causing the movement of the Cl^- ions may be set simply five times as great as the driving force for K^+. It is probable that the figure five is not quite correct, because neither osmotic pressure nor activity is exactly proportional to the concentration. However, these deviations are certainly within the ranges of the other errors in these experiments.

In this way, the "specific mobility" of K^+ and Cl^-, or at least the ratio of the two, is calculated in the last column of Table V. It may be emphasized that these ratios hold only for the conditions of this experiment, since the specific mobility of an ion within the membrane depends greatly on the conditions, especially on the concentration, to a much greater extent than it does in a free aqueous solution.

SUMMARY.

The theoretical aspects of the problem of sieve-like membranes are developed.

The method of preparing the dried collodion membrane is described, and the method of defining the property of a particular membrane is given. It consists of the measurement of the *Co P*, that is the P.D. between an 0.1 and an 0.01 M KCl solution separated by the membrane. *Co P* is in the best dried membranes 50 to 53 millvolts, the theoretically possible maximum value being 55 millivolts. Diffusion experiments have been carried out with several arrangements, one of which is, for example, the diffusion of 0.1 M KNO_3 against 0.1 M NaCl across the membrane. The amount of K^+ diffusing after a certain period was in membranes with a sufficiently high *Co P* (about 50 millivolts or more) on the average ten times as much as the amount of diffused Cl^-. In membranes with a lower *Co P* the ratio was much smaller, down almost to the proportion of 1:1 which holds for the mobility of these two ions in a free aqueous solution. When higher concentrations were used, *e.g.* 0.5 M solution, the difference of the rate of diffusion for K^+ and Cl^- was much smaller even in the best membranes, corresponding to the fact that the P.D. of two KCl solutions whose concentrations are 10:1 is much smaller in higher ranges of concentration than in lower ones.

These observations are confirmed by experiments arranged in other ways.

It has been shown that, in general, the diffusion of an anion is much slower than the one of a cation across the dried collodion membrane. The ratio of the two diffusion coefficients would be expected to be calculable in connection with the potential difference of such a membrane when interposed between these solutions. The next problem is to show in how far this can be confirmed quantitatively.

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PHOSPHATE ION AS A PROMOTER CATALYST OF RESPIRATION.

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I.

In other papers (Lyon, 1923–24, 1927) results have been reported which appeared to show that neutral solutions of sodium or potassium phosphate serve to catalyze the enzymatic production of CO_2 by plant tissues. The oxidising enzymes involved were those of *Elodea canadensis*, wheat seedlings, and potato tubers, the latter studied only in aqueous extracts. We shall now present additional proof of this promoter action through more careful analyses to determine the active component of mixtures of mono- and disodium phosphates.

The molar concentrations of the solutions which gave optimum results were somewhat high for the usual types of catalysis. The concentration most used was approximately 0.1 M, by which is meant a solution obtained by mixing 0.1 M monosodium phosphate with 0.1 M disodium phosphate. The complex nature of the components of such a solution suggested that some single element among them was the active, or at least the controlling factor of the catalysis. Since the ionization of even this concentration of the sodium phosphates is presumably complete, we are concerned primarily with the nature of the ionization products of phosphoric acid. An excellent statement of the conditions of equilibrium between H_2PO', HPO_4'', and PO_4''' is given by Holt, La Mer, and Chown (1925[1]), from which it is apparent that for H_3PO_4, K_1 is very large, K_2 is smaller, and K_3, which determines the relative amounts of PO_4''', is very small. These authors have also calculated the concentrations of PO_4''' in relation to pH over a wide range and have introduced the expression $p[PO_4''']$ which may

[1] Holt, La Mer, and Chown (1925) pp. 518 to 522.

be used to express the concentration of this ion just as $p[H^+]$ is used for the hydrogen ion.

The relative concentration of PO_4''' is so low that it must be admitted that it is no higher than that of well known catalysts such as the H^+ ion. At pH 7.0 only 1/500,000th of the total P present is present in this ion. On the alkaline side of neutrality the relative concentration of PO_4''' increases rapidly and on the acid side it falls off. A plot of tables of Holt, La Mer, and Chown (1925[2]) shows that the graph of $p[PO_4''']$ (we prefer to use the form pPO_4) against pH is not a straight line but a curve between pH 6 and 9, which correspond to pPO_4 7.44 and 3.4, respectively. Thus pPO_4 will vary with both pH and the molar concentration of the acid or its sodium salts.

Similar statements could be worked out for the other ions of the solutions but it is this ion which proves to be related to catalysis through mathematically simple and exact rules.

As we shall show later, the concentration curve for the rate of CO_2 production by *Elodea canadensis* (Lyon[3]) in different concentrations of neutral phosphate solutions after an exposure of 1 hour does not afford the best data by which to test the conception of catalysis by the PO_4''' ion. At the two extreme concentrations other factors intervene to mask the real effect. When the concentration is low the element of rate of penetration of the phosphate into the living cells limits the observed effect at the end of 1 hour. At the higher concentration there is opportunity for a deleterious effect through either the osmotic effects or some other result of the presence of such a high concentration of salts. The intermediate data are too few to use.

Accordingly, we have performed the experiments necessary to provide data for the effect of change of pPO_4 through change of pH on the alkaline side of neutrality. Similar experiments were attempted for the acid side, but the presence of carbonates or bicarbonates gave rise to such an increase in the liberation of CO_2 that such readings are not comparable with those at a higher pH. In Fig. 1 are shown individual time curves of experiments on the alkaline side of neutrality. The significant values for our purpose are the levels at which each curve flattens out. These experiments were performed according to

[2] Holt, La Mer, and Chown (1925), p. 521.

[3] Lyon (1923–24), p. 302.

the technique described in previous papers, the main apparatus being a suitable form of the Osterhout respirometer (Osterhout, 1918–19, 1919–20). Hence, the preliminary rise in most of these curves is

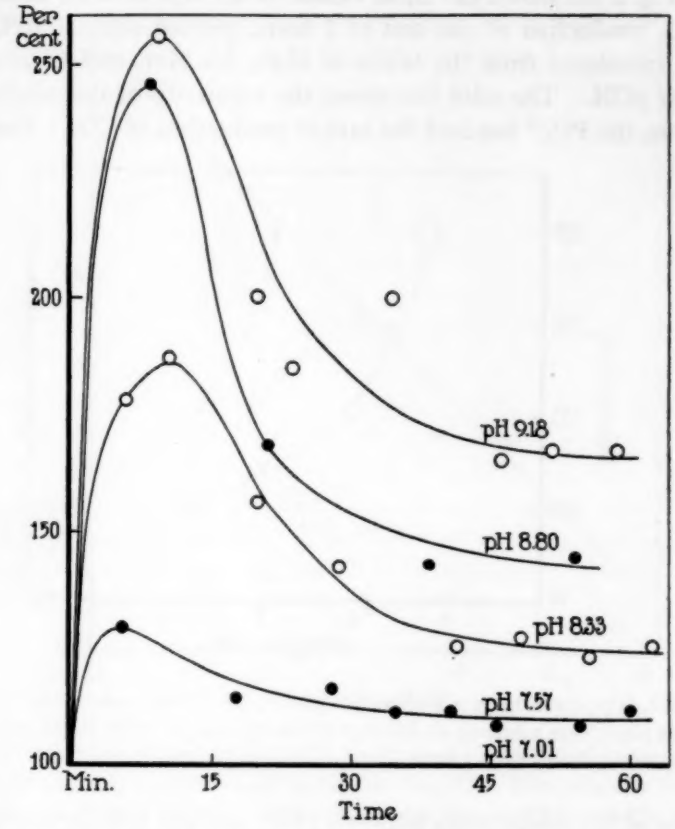

FIG. 1. The effect of alkaline phosphate solutions on the production of CO_2 by *Elodea canadensis*. Each curve represents a typical experiment in which the normal (taken as 100 per cent) is obtained with an 0.106 M neutral phosphate solution and followed by the application of phosphate mixtures of the same molar concentration but with the pH as indicated for each curve.

probably not due to a serious error such as the introduction of atmospheric CO_2 at the time of application of the alkaline solution. It is more likely that we have to do with a temporary change in equilibrium

within the system though there may possibly be a real temporary increase in rate of CO_2 production above that at which a level is attained.

In Fig. 2 are shown the mean values (of all experiments) of the rate of CO_2 production at the end of 1 hour, plotted against pPO_4, the latter calculated from the tables of Holt, La Mer, and Chown, and against pOH. The solid line shows the regularity of the relationship between the PO_4''' ion and the rate of production of CO_2. From the

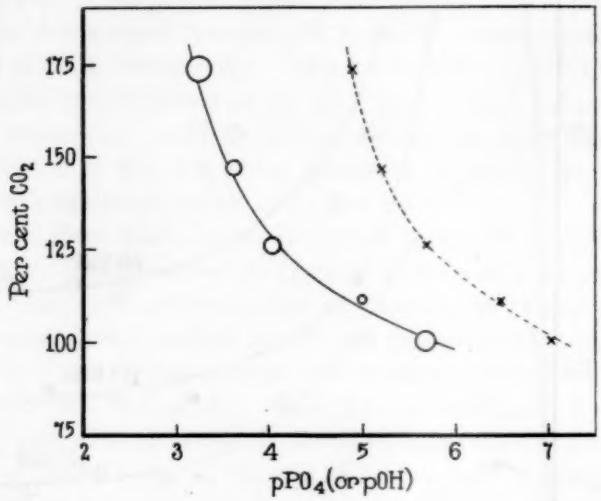

FIG. 2. The mean rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4 (solid line) and to pOH (broken line), after 1 hour.

nature of the relationship between pPO_4 and pH it is to be expected that the relationship to pOH (broken line) should also be regular. We are attempting to show that the principal effect is that of the PO_4''' ion rather than the OH^- or H^+ ion.

The curve for the relationship to the PO_4''' ion resembles that of a hyperbola with the general equation (for these coordinates)

$$(CO_2 - a)\,(pPO_4 - b) = K,$$

where a and b represent the fact that the asymptotes of this hyperbola may not be $CO_2 = 0$ and $pPO_4 = 0$ but $CO_2 = a$ and $pPO_4 = b$. The mathematical solution of this equation for the five measured points on

the curve was accomplished by a reliable method that depends upon the general method of least squares. The equation which results is

$$(CO_2 - 68.475)\ (pPO_4 - 2.13) = 114.43.$$

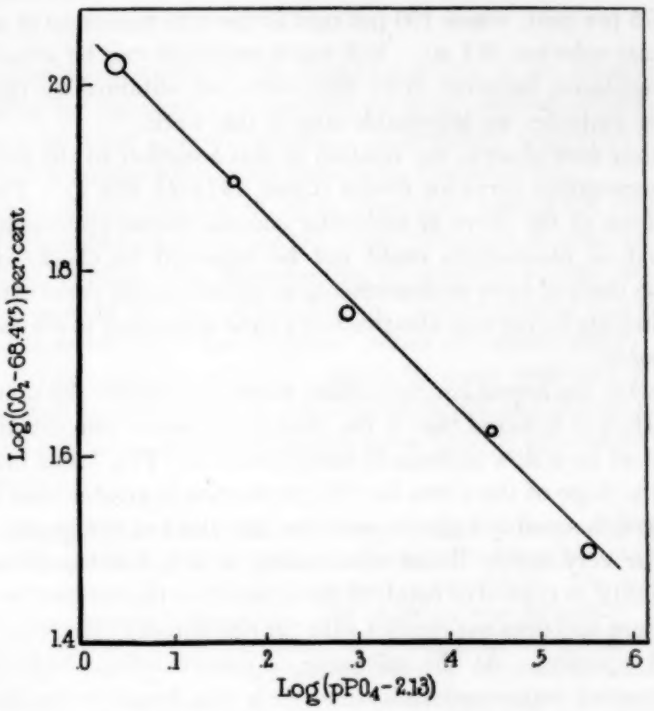

FIG. 3. The rates of production of CO_2 by *Elodea canadensis* in 0.106 M alkaline phosphate solutions of different pH in relation to pPO_4. The ordinates are logarithms of per cent $CO_2 - 68.475$ while the abscissæ are logarithms of $pPO_4 - 2.13$. The straight line is the plot of values calculated from the equation in the text.

The closeness of fit to the data may be seen in Fig. 3 in which the logarithm of the per cent CO_2 is plotted against the logarithm of pPO_4. If the hyperbolic relationship holds the points should lie in a straight line in this type of plot. The straight line we have drawn represents the calculated equation while the points indicate the locations of the measurements.

The meanings of the constants a and b are interpreted as follows: Under the conditions by which the relationship was derived (*viz.* with

the pH also increased) the one asymptote locates the point beyond which further increase in concentration of PO_4''' ions fails to give an increase in rate of CO_2 production; the other asymptote denotes the fact that the enzyme may function apart from PO_4''' ions to the extent of 68.475 per cent, where 100 per cent is the rate measured in neutral phosphate solution (0.1 M). Not much emphasis can be attached to these constants, however, since they were not obtained by changing only one variable—an impossible step in this work.

We may now observe the relation of this equation to the points on the concentration curve for *Elodea* (Lyon, 1923–24, Fig. 2). The horizontal form of the curve at molecular concentrations approaching the threshold of plasmolysis could not be expected to check with an equation derived from measurements at optimum salt concentrations. Some limiting factor may also prevent a close adherence to a hyperbolic relationship.

Likewise, the lowest concentrations might not exhibit the same relation. Here it is found that if the increase in molar concentration be thought of as a slow increase in concentration of PO_4''' ions (as is the case), the slope of the curve for CO_2 production is greater than that of Fig. 2 and is sensibly uniform over the first third of the graph. This linear, or very nearly linear relationship, which denotes direct proportionality, is typical of catalytic processes over the range of low concentrations and does not conflict with the relationship expressed by our derived equation. At the midrange of concentration, however, we should expect some conformity and such was found to be the case. The total difference between the K's computed for the two known midpoints is only 1.5 per cent of the mean K.

This is as far as we can go in the analysis of our own data which seem to point to the PO_4''' ion as the effective catalyst of oxidising enzymes. It would be very desirable to obtain similar concentration curves for other plant material, by experiments involving change of concentration of the phosphate ion by changing first the pH and then the molar concentration. We plan to conduct such studies at a later date.

The results of Bode (1926), who sought to measure the dependence of respiration upon hydrogen ion concentration, show a qualitative agreement with our results in so far as his data may be converted into data for pPO_4. His use of calcium and magnesium phosphates to regulate the pH introduces new variables affecting the exact concen-

tration of the phosphate ion and involving the influence of the metal. In general, however, the presence of higher concentrations of phosphate ion correlates with a higher rate of respiration as measured by the absorption of oxygen.

II.

A further confirmation of the promoter action of the phosphate ion is afforded by the results of analyses of some existing records in the literature of other enzyme actions. Appropriate treatment of such records demonstrates the same hyperbolic relationship.

The widespread practice of using phosphate buffers for controlling the pH of enzyme reactions has led to the statement of some records in a form which allows the transposition of either molecular concentration or change of pH into data for pPO_4. Unfortunately it is common practice to report but a few "typical" series of measurements. Mean values of two measurements were found for some cases and of course these as well as data covering both change of pH and change of molar concentration at constant pH in the same type of experiments carry more weight.[4]

The method of procedure in the conversion of data into terms of pPO_4 will be described for only the first set of readings to be dealt with—those given by Smirnoff (1925) in a study of the effect of neutral salts on peroxidase. The enzyme was obtained from ground wheat seeds. The substrate was pyrogallol. The criterion of enzyme action was the amount of purpurogallin formed, estimated by titration with $KMnO_4$. The data for the effect of the phosphate solution of concentration N/80 were given for only one set of experiments, in the following tabular form.

pH	3.5	4.0	4.98	6.5	7.1	7.5	7.86	8.3
Activity of enzyme, per cent.	1.9	2.84	55.79	100.0	135.0	148.16	150.53	175.07
pH	8.7		9.3	9.5				
Activity, per cent.	183.64		173.86	139.68				

[4] The measurements, however, are all recorded in terms of amounts of product after equal periods of time. That this is not always the proper measure of enzyme action was shown in the recalculation by Northrop (1924-25) of the results of Morgulis (1921) in a study of the kinetics of catalase action on peroxide.

From these data it is obvious that below pH 4.98 there is a powerful inhibition of enzyme action and above pH 8.7 there is a depressive action. These effects are undoubtedly due to a specific effect of the pH value and we can only use the intermediate data. The necessary data for a logarithmic plot in terms of pPO_4 is given in the following table. The pPO_4 was obtained by interpolation from a plot of the table of pPO_4 for various pH values.

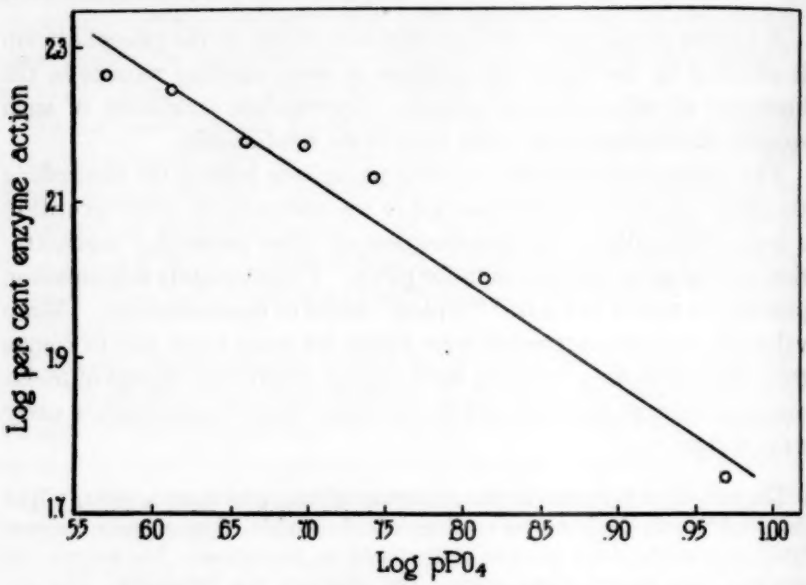

FIG. 4. The logarithmic plot of the relation of the concentration of PO_4''' ions to the percentage rate of oxidation of pyrogallol to purpurogallin by peroxidase. The straight line is the plot of the equation

$$(\text{Activity of enzyme})\ (pPO_4)^{1.34} = K.$$

The indicated points represent a single set of readings as reported by Smirnoff (1925).

pH	pPO_4	Log pPO_4	Enzyme activity in per cent	Log enzyme activity
4.98	9.43	0.9745	55.79	1.7465
6.5	6.51	0.8136	100.0	2.0000
7.1	5.53	0.7427	135.0	2.1303
7.5	5.00	0.6990	148.16	2.1707
7.86	4.58	0.6609	150.53	2.1775
8.3	4.10	0.6128	175.07	2.2430
8.7	3.72	0.5705	183.64	2.2640

Fig. 4 shows the plot of these values. The points lie as near to a straight line as could be expected for a single set of readings. The slope of this line is not -1, however, but -1.34. Therefore the equation is

$$(\text{Activity of enzyme})\ (pPO_4)^{1.34} = K.$$

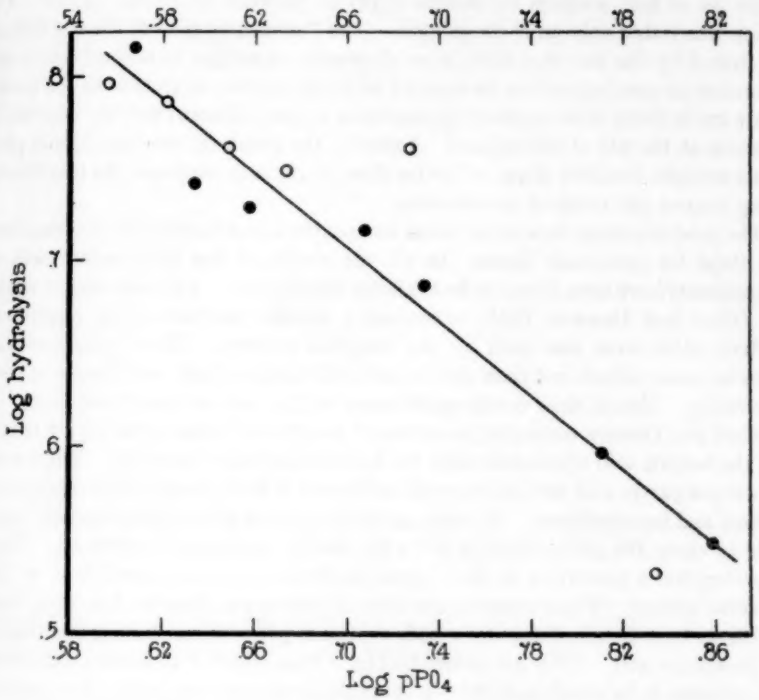

FIG. 5. Logarithmic plot of the relation of concentration of PO_4''' ions to the rate of hydrolysis of ethyl butyrate by pancreatic lipase in glycerin extracts of the pig pancreas. Data are taken from the work of Platt and Dawson (1925) who varied pPO_4 by change of pH. The straight line is fitted to the points (●) obtained from the readings for an experiment involving the use of low concentration of phosphate salts. The abscissæ are those from the scale at the bottom of the figure and the equation for the line is

$$(\text{Hydrolysis})\ (pPO_4)^{1.01} = K.$$

The other points (○) are referred to the scale of abscissæ at the top of the figure, this scale differing from the lower one only by a displacement to the right by four units. These points were taken from a second experiment which differed only in the use of five times as much phosphate salts in the buffer solutions.

Smirnoff did not study in sufficient detail the effect of the concentration of phosphate solutions to warrant a similar test of the phosphate ion relations at constant pH. The method of calculation of pPO_4 under those conditions is fundamentally the same and will appear in connection with lipase analyses.

For another of the oxidising enzymes—laccase from alfalfa—we have found a single set of four readings for change of pH as recorded by Bunzel (1915). Its action was tested only on hydroquinone. The "unnaturalness" of the conditions is attested by the fact that there is no absorption of oxygen in neutral solutions. Therefore no conclusions can be reached as to the relation of laccase to the phosphate ion in living cells in which the substrate is quite different and the enzyme is effective at the pH of protoplasm. Actually, the graph on the logarithmic plot was a straight line with slope -7.46 for three of the four readings, the fourth one being nearest the point of no oxidation.

The most complete data to be found for enzyme action in relation to phosphate are those for pancreatic lipase. In all, the results of five independent sets of experiments have been found to be available for analysis. The most recent workers (Platt and Dawson, 1925) recognized a specific function of the phosphate buffers which were also used by the previous workers. Their technique was likewise more refined and their data cover both change of pH and change of concentration. Hence, their results carry more weight and are considered first.

Platt and Dawson estimated the action of pancreatic lipase of the pig by titrating the butyric acid released through the hydrolysis of ethyl butyrate. They were concerned partly with the optimum pH and found it to be about 7.0 for phosphate buffers and purified lipase. By using glycerin extracts of the pancreas they were able to carry the pH as high as 8.0 with steadily increasing hydrolysis. They consider that a protection of the enzyme is afforded by some constituent of the glycerin extract. These experiments (Nos. 5 and 6) are therefore the ones from which we have obtained data for a wide change of pH with constant concentration of phosphate salt. They are shown in Fig. 5 from which it is evident that their Experiment 5, in which only 5 cc. of phosphate solution was used, gives an unbroken straight line through a wide range of pPO_4. Experiment 6 involved the use of 25 cc. of phosphate solution and the points (shown by ○) are somewhat irregularly distributed. In both experiments the points represent single readings and additional experiments would probably remove the irregularities. The general trend of the points is not far from that for the lower concentration of salt, the equation for which is

$$(\text{Hydrolysis})\ (pPO_4)^{1.01} = K.$$

After a specific effect of phosphate was observed the point was carefully studied by using different molar concentrations of the buffer salts (*no hydrolysis could be observed in the absence of phosphate*). In the first of these studies (their Experiment 9) low concentrations were employed at pPO_4 4.9 (pH 7.6). The results show that for the range 0.005 to 0.05 M there is a hyperbolic relationship between the

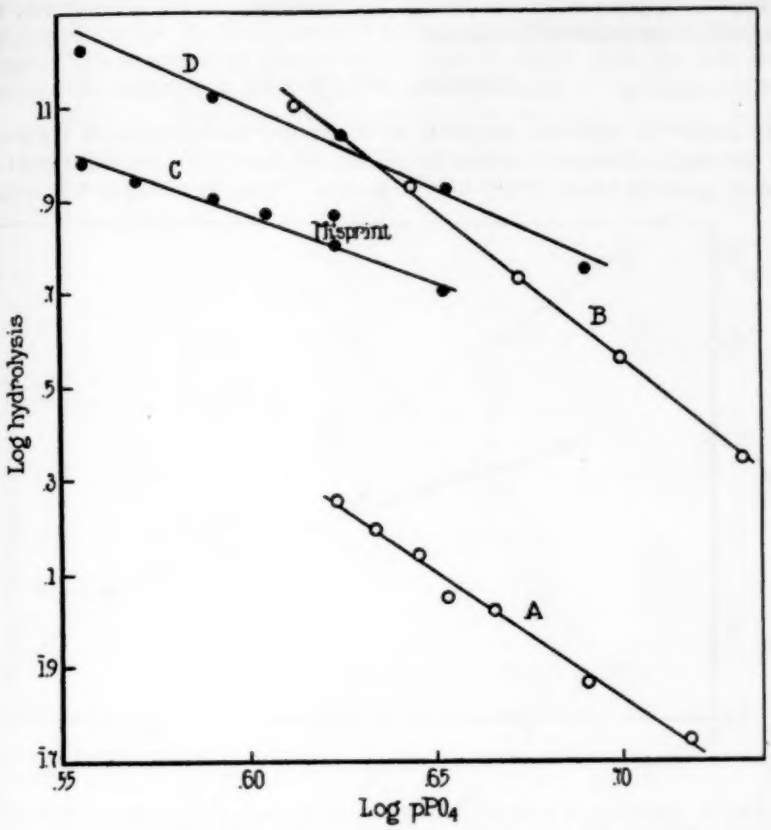

FIG. 6. Logarithmic plot of the relation of pPO_4 to the rate of hydrolysis of ethyl butyrate by pancreatic lipase. Data are taken from the work of Platt and Dawson (1925) in which the pPO_4 was varied by the use of varying concentrations of phosphate salts at constant pH. Each point represents the mean value of two readings. The equations for the lines, together with the pH used for each, are as follows:

Curve *A* (Hydrolysis) $(pPO_4)^{5.64} = K$ pH = 7.6
" *B* (Hydrolysis) $(pPO_4)^{6.32} = K$ pH = 7.2
" *C* (Hydrolysis) $(pPO_4)^{2.9} = K$ pH = 7.6
" *D* (Hydrolysis) $(pPO_4)^{3.59} = K$ pH = 7.6

The misprint indicated in connection with Curve *C* was obviously a mistake in one figure of the number given in the original paper.

amount of hydrolysis and the pPO_4. The logarithmic plot is given in Fig. 6, Curve *A*, which indicates the relation

$$(\text{Hydrolysis})\ (pPO_4)^{5.64} = K.$$

The method of obtaining the pPO_4 for the various concentrations is the same as that used in similar conversions for pH and was done by the use of the table of factors given by Clark (1922[5]) for this purpose. Thus the pPO_4 at 0.01 M (all

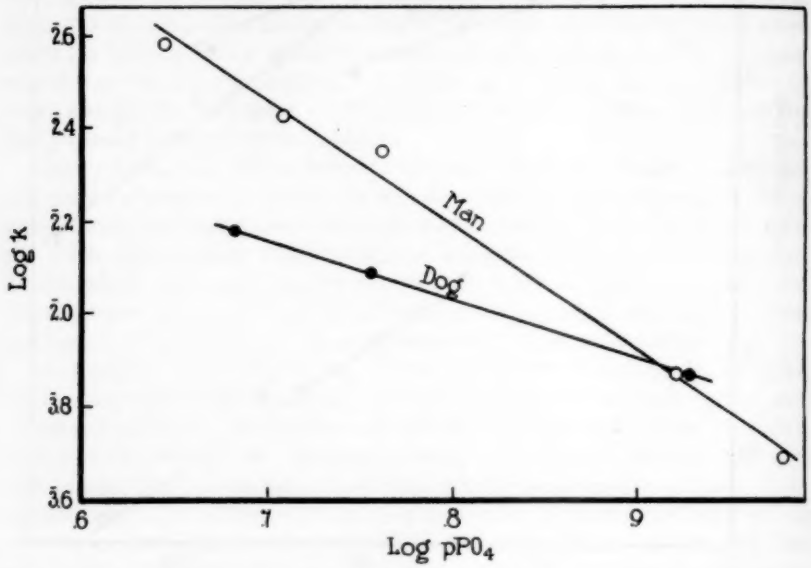

FIG. 7. Logarithmic plot of the relation of pPO_4 to the action of pancreatic lipase on tributyrin. Data are taken from the work of Rona and Pavlović (1922). The pPO_4 was varied by using buffer solutions of pH 4.94 to 8.01. The equations for the lines are

$$\text{for lipase from dog } (\bullet) - K\ (pPO_4)^{1.26} = K$$
$$\text{“ “ “ “ } (\circ) - K\ (pPO_4)^{2.7} = K$$

Each set of points is taken from a single experiment.

calculations were corrected to this concentration) is 4.9 while that at 0.03 M is 3×4.9 or 0.3×3.9 which is pPO_4 4.42.

In Fig. 6 there are also shown the results of the remainder of the tests with change of concentration at constant pH. For the experiment shown by Curve *B* the pH was 7.2. This is nearer the optimum than was the case for Curves *C* and *D* in which the pH was 7.6. Platt and Dawson comment on the difference in

[5] Clark (1922), p. 456.

form between these two types of curves as plotted using molar concentration, and say that the farther the pH is above the optimum, the more pronounced the curvature. This is somewhat noticeable in Curves *C* and *D* when the data are converted to a logarithmic plot. However the conditions in the experiments were

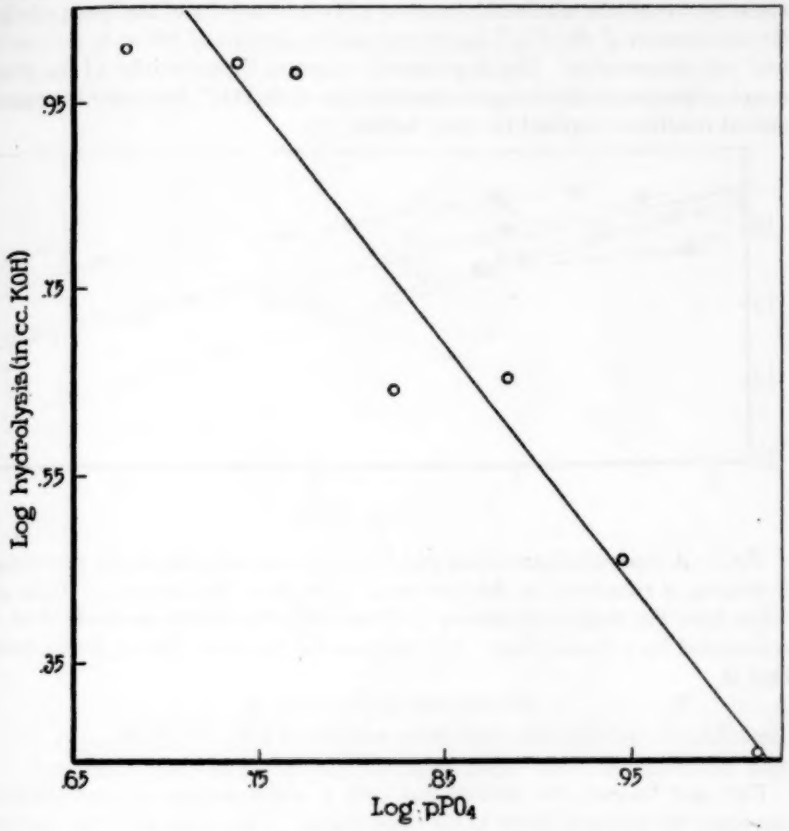

FIG. 8. Logarithmic plot of the relation of pPO_4 to the hydrolysis of olive oil by pancreatic lipase of the pig. Data are taken from a single experiment by Umeda (1915). The pPO_4 was varied by the use of buffer solutions of pH 4.494 to 7.731. The equation for the line as drawn is

$$(\text{Activity of enzyme})\ (pPO_4)^{2.54} = K.$$

essentially the same as for those shown by Curve *A*. (This was also our justification for suggesting the indicated misprint in the data used for Curve *C*; see legend).

The equations for the four experiments in which the pPO_4 was altered by change of molar concentration are given in the legend of Fig. 6. They differ from the

equation for the same relationship derived by change of pH only in the slope of the line, which is also indicated by the exponent of pPO_4 in the equation. This difference is directly attributable to the attendant differences in molar concentration and hydrogen ion concentration. The straight lines on the logarithmic plots attest the hyperbolic relationship between pPO_4 and activity of the lipase, *whether the concentration of the PO_4''' ion be increased by decrease of pH or by increase of total salt concentration.* The slope merely measures the sensitivity of the given sample of enzyme to the change in concentration of the PO_4''' ion under the experimental conditions imposed by other factors.

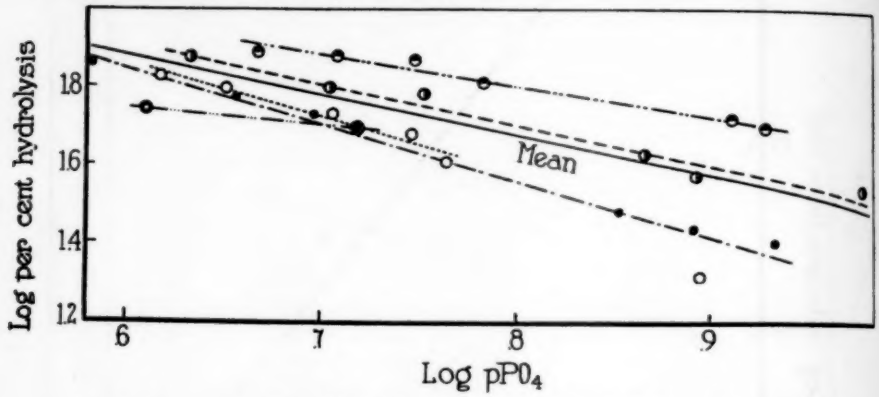

FIG. 9. A composite logarithmic plot of the relation of pPO_4 to the percentage hydrolysis of tributyrin by the pancreatic lipase from beef pancreas. Data are taken from five single experiments by Rona and Bien (1914), each of which is represented by a broken line. The equation for the mean line as drawn (solid line) is

$$(\text{Hydrolysis})\ (pPO_4)^{1.08} = K.$$

The pPO_4 was varied by the use of buffer solutions of pH 4.87 to 8.58.

Platt and Dawson also noticed that both *a*- and *b*- sodium glycerophosphate promoted the action of lipase to the same degree. They state their opinion that this indicates an effect by the phosphate ion. The present analysis confirms this observation.

Another recent measurement of the action of pancreatic lipases (purified) on tributyrin is reported by Rona and Pavlović (1922). The available data are very meager but the uniformity of the results is evident from the form of the plots in Fig. 7. Only three measurements are available for the lipase from dog pancreas and there are but five for that from the human pancreas. Each set of points, however, lies along a straight line, the slope of which differs for the two types of enzymes. The "*k*" used as a measure of the activity of the enzyme was calculated by the authors from a monomolecular equation for the hydrolysis. In each case the pPO_4 was altered by change of pH.

The work of Umeda (1915) furnished a single set of readings of the hydrolysis of olive oil by purified pancreatic lipase of the pig, in relation to pPO_4 as calculated from the pH. The lipoclasis was estimated by titration of the acid produced after 20 hours. Fig. 8 shows the same hyperbolic relationship to pPO_4. Here the equation is

$$(\text{Activity of enzyme})\ (pPO_4)^{2.54} = K.$$

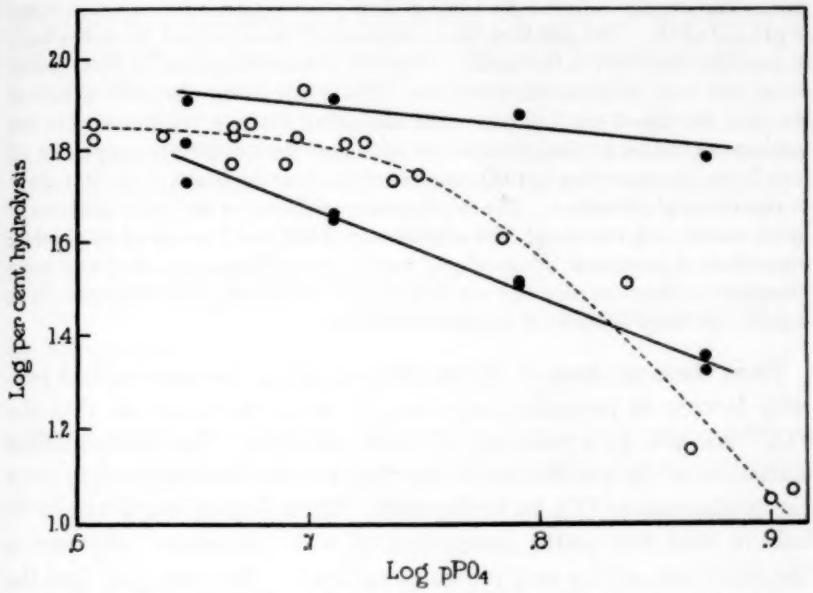

FIG. 10. Logarithmic plot of the relation of pPO_4 to the percentage hydrolysis of fat by pancreatic lipase of the pig (shown by (●) and solid lines) and by lipase of the duodenal juice (shown by (○) and broken line). Data are taken from single readings by Davidsohn in 1913.

Rona and Bien (1914) used a glycerin extract of lipase from beef pancreas and estimated the hydrolysis of tributyrin by a stalagmometric method. This method is not so accurate as titration. The results are converted into percentage of hydrolysis and plotted against pPO_4 as usual in Fig. 9. This is a composite of several experiments, each of which is indicated by a broken line. The variation from the average line is due largely to the variation in the activity of the sample of enzyme used. Each experiment gave a straight line plot and the mean slope is -1.08. Therefore the equation is the same as those derived from later measurements.

In Fig. 10 are shown the results from a still earlier report (Davidsohn, 1913) of hydrolysis of fat by pancreatic lipase of the pig. This is the only case we have

found in which all the points do not lie along a straight line on this type of plot. The two solid straight lines in the figure are drawn in respect to the readings from three experiments with lipase direct from the pancreas, one sample of which was obviously more active than the other two. These data clearly exhibit the same type of relationship as has been demonstrated in the previous analyses.

The other points ($\overline{\text{o}}$) on this composite plot represent data from a number of single measurements with lipase contained in the duodenal juice. The curved, broken line roughly indicates the trend of these points as plotted over a wide range of pH and pPO_4. The points at the extremes could be accounted for on the basis of the unfavorable pH of the media. However, the distribution of all these points is not only very irregular but uncertain. This is due to the composite nature of the data, the use of the stalagmometric method of analysis, the variation in the total concentration of phosphate buffer salts, and the inability to correct for all such factors in converting to pPO_4 because of the incompleteness of the statement of experimental procedure. The relationships exhibited by our other analyses of lipase studies and the recent demonstration by Platt and Dawson of an absolute dependence of pancreatic lipase, also of the pig, upon the presence of at least some phosphate, appears to outweigh the doubtful evidence from these older results as regards the lipase obtained in a different medium.

From these analyses of the relation of lipase, peroxidase, and possibly laccase to phosphate solutions, it seems quite certain that the PO_4''' ion acts as a promoter of their activities. The mathematical statement of the relationship is like that for the effect of phosphate on the production of CO_2 by living cells. There is then every reason to believe that the active component of such phosphate solutions is the PO_4''' ion, acting as a promoter catalyst. The very fact that the mathematical expression of the relationship is of the form

$$(\text{Activity of enzyme})\ (pPO_4)^n = K$$

is of itself an additional proof; for the term "pPO_4" is a direct measure of the potential of the PO_4''' ion in a given solution. The inverse proportionality expressed by the equation is really a direct proportion because of the peculiar method of statement of the potential.

In the case of plant respiration the exponent of the pPO_4 term was found to be 1. The corresponding exponent in the case of peroxidase was essentially the same (1.34). Although an exponent of this order was also found for a few cases in the lipase analyses (*cf.* Figs. 5, 7, and 9), the value 3 or 6 was more characteristic of lipase (*cf.* Figs. 6 to 8). The agreement of the numbers of the "oxidase" group of enzymes is significant while the question of sensitivity of lipase (measured by

the value of the exponent, n) to the phosphate ion is beyond the immediate scope of our problem.

III.

SUMMARY.

The active component of phosphate solutions, in relation to promoter action on oxidising enzymes, is the PO_4''' ion. This is shown by the demonstration of a hyperbolic relationship between per cent production of CO_2 (of *Elodea*) and pPO_4, the measure of the phosphate ion potential. This is consistent with the rate of respiration as affected by changing pPO_4 through change of total phosphate concentration while pH is kept constant. The equation for this relationship is

$$(CO_2 - a)\ (pPO_4 - b)^n = K$$

where a, b, n, and K are constants and $n = 1$.

The same relationship to phosphate ion concentration, expressed by the equation

$$(\text{Activity of enzyme})\ (pPO_4)^n = K,$$

where n and K are constants and n varies from 1 to 6 under different conditions, appears to hold for some other enzyme actions, including those of peroxidase and pancreatic lipase.

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ARSENATE AS A CATALYST OF OXIDATION.

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In connection with another paper describing the catalytic rôle of phosphate in plant respiration (Lyon, 1927) brief mention is made of some experiments in which the effect of arsenate on plant respiration and on oxidation was observed qualitatively. A catalytic effect is found in both cases. The importance of this hitherto unconsidered property of arsenate seems to justify a more detailed report of experiments which have been extended in order to provide more data.

It should be borne in mind that the present discussion of the catalytic effect of arsenate does not detract from the apparently unique rôle of phosphate as a non-toxic promoter catalyst for the respiratory enzymes. The same concentration of arsenate which momentarily increases the rate of production of CO_2 by living tissues soon exercises a toxic effect.

There are several suggestive items of evidence to be found in the literature regarding the effect of arsenate salts. Most of those to which we shall refer have resulted from comparisons of arsenate with phosphate in models or isolated phases of respiration. These are therefore particularly valuable items of evidence.

In the oxidation-reduction system of Haehn and Pülz (1924) a supposed cleavage of water molecules is effected by means of a combination of amino acid and phosphate. At least the presence of these two reagents in aqueous solution results in an oxidation of acetaldehyde and a reduction of methylene blue, at relatively high temperatures. Arsenate was found to be equally effective when substituted for the phosphate.

Meyerhof and Matsuoka (1924) have repeated and extended the observations of Warburg and Yabusoe (1924) on the oxidation of fructose by pure oxygen in a solution of phosphates presumably containing iron salts. They found that arsenates could be used in place of phosphates without destroying the effectiveness of the iron catalysis.

The substitution of arsenate for phosphate in alcoholic fermentation cannot be made to the extent of a complete replacement of phosphate. Some phosphate is

essential to the formation of hexose phosphate, an ester of hexose and potassium phosphate. This ester is continually being formed and decomposed by certain enzymes. Harden and Young (1911) did observe that arsenate could replace phosphate in the sense that the latter causes an increase in the production of CO_2 (and alcohol). They attributed this effect to an increased activity of hexose phosphatase, the enzyme which splits the ester.

When Harden and Henley (1922) reviewed the work of Witzemann (1920) on the oxidation of glucose by H_2O_2 as affected by phosphate, they were inclined to the view that the buffered solution of phosphates aided the reaction by the effect on the peroxide. They demonstrated a qualitatively similar action by other salts such as carbonates. However, their results show a special effect of phosphates and arsenates over and above the buffer action. This effect has never been clearly explained.

Our first experiments with arsenates consisted in adding them to slowly oxidizing solutions of pyrogallol and to slowly respiring tissues of *Elodea canadensis*.

Whenever a few drops of arsenate solution—either the alkaline (pH = 9.18) solution of disodium arsenate or the same solution brought to pH. 7.0 with the same molar concentration of arsenic acid—are added to a relatively large volume of a solution of pyrogallic acid there is a rapid coloration of the solution. This indicates an oxidation of the pyrogallol, the end-products being purpurogallin, carbon dioxide, and water. It is a matter of hours before the usual slow oxidation forms sufficient purpurogallin to give a pale straw color to the solution, but the addition of arsenate provides the same color in not more than 2 minutes. The effect of arsenate is qualitatively the same as that of phosphate (*cf.* Lyon, 1927).

In a typical experiment 5 drops of a 0.085 M solution of disodium arsenate are added to 15 or 20 cc. of 1 per cent solution of pyrogallol. For the control there is added the same number of drops of a solution of NaOH which has been diluted until the hydroxyl ion concentration is the same or greater than that of the arsenate solution. The difference in color between the experiment and control increases rapidly during the 1st hour or 2 and is more marked near the surface of the solution. The difference is maintained for days and weeks while both solutions become yellowish, then brown, and finally brownish-purple. No previous mention of such an effect has been found.

In Fig. 1 are shown six individual time curves of the rate of production of CO_2 by *Elodea canadensis*. These experiments were carried out

according to the technique described in a previous paper (Lyon, 1923-24), using a form of the apparatus described by Osterhout (1918–19). The normal rate of production of CO_2 by the untreated tissues is taken as 100 per cent. The subsequent changes in the rate are indicated by a curve smoothed through points each of which represents the rate measured at a certain time after the addition of a sufficient volume of

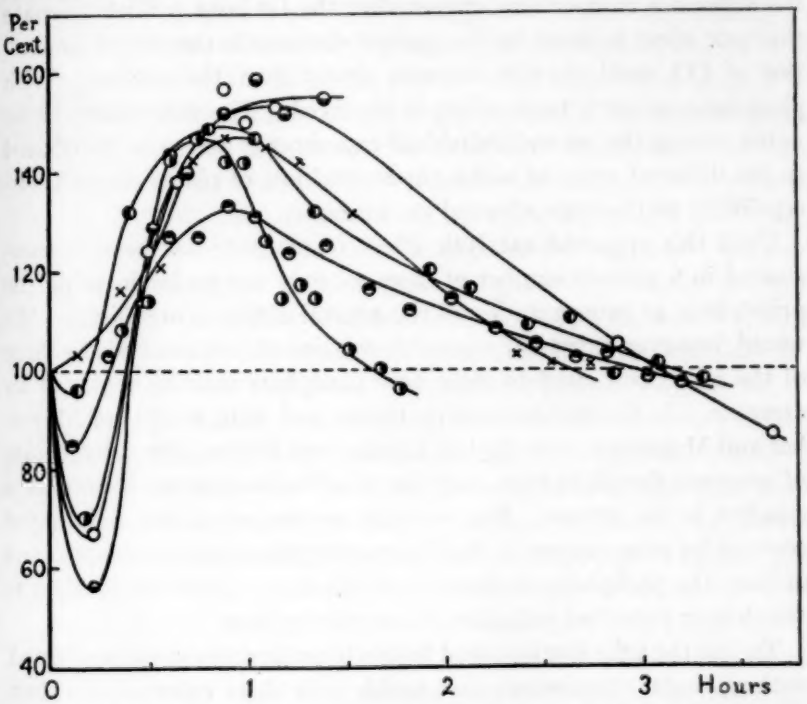

FIG. 1. The effect of 0.1 M neutral arsenate solutions on the production of CO_2 by *Elodea*. Each curve represents a single experiment. The normal rate before the addition of arsenate is taken as 100 per cent.

a concentrated solution of neutral mixtures of disodium arsenate and arsenic acid to give an effective concentration of 0.1 M.

The fall of the first readings below normal is to be regarded as the result of the increased CO_2-absorptive capacity of the diluted solutions of arsenates (*cf.* Fig. 1 in Lyon, 1923–24). The rise of each curve above the 100 per cent level shows the manner in which the rate of

production of CO_2 is uniformly increased after about 1 hour. The similarity of this part of each curve with that of the individual curves for the effect of phosphate (*loc. cit.*) is evident. This close parallel may lead us to believe that arsenate also acts as a catalyst to the respiratory enzymes of *Elodea*.

The difference in the effects of arsenate and phosphate is shown by the difference in their time curves after the 1st hour. With arsenate the toxic effect is shown by the gradual decrease in the rate of production of CO_2 until the rate becomes slower than the normal. With phosphate no such toxic effect is observed. The differences to be noted among the several individual experiments are to be attributed to the different rates at which the several lots of plants showed susceptibility to the toxic effect of the arsenate.

Until this apparent catalytic effect of arsenate has been demonstrated in a greater number of cases we may not go far in using the principle in an interpretation of the general action in organisms. We would, however, point out a possible relation of this catalysis to three of the four cases cited to show how phosphate may be replaced by arsenate. In the system used by Haehn and Pülz, in that of Meyerhof and Matsuoka, or in that of Harden and Henley, the substitution of arsenate for phosphate may be possible because each acts as a catalyst in the system. For example, in the case of the oxidation of fructose by pure oxygen in the presence of phosphate or arsenate and of iron, the phosphate or arsenate may act as a promoter catalyst to the slow or potential oxidation of fructose by iron.

To test the principle involved in this hypothesis we have performed, with arsenate, experiments comparable with those reported elsewhere for phosphate (Lyon, 1927). Into each of four tubes were placed 15 cc. of a 1 per cent solution of pyrogallol. Additions of substances selected for an analysis of their effect on the rate of oxidation were made as follows: (1) 4 drops of 0.34 M neutral arsenate mixture; (2) the same plus a small, clean, iron nail; (3) 4 drops of water plus a similar iron nail; (4) 4 drops of water. No. 4 was a control and the rate of oxidation was so slow that no important color change could be observed for hours. The arsenate in 1 induced a visible production of color after not more than 2 minutes. Essentially the same color appeared in 2 above the nail and even less color finally came in 3

above the nail than in 4. But at the surfaces of the iron there appeared strongly colored regions. The region in 2 was much greater in volume than that in 3 but was violet-purple in contrast with the pure purple about the iron in 3.

During the remainder of such an experiment the tubes were frequently stirred in order to avoid gradients in the concentrations of oxygen and oxidation products throughout the solution, particularly in the vicinity of the iron. This procedure caused no change in the apparent color of the oxidation products, but after 2 to 4 hours it was observed that the density of the coloration in No. 2 was much greater than that in either 1 or 3. To determine whether this was anything more than the additive effect of the two catalysts, the color was compared with that of a mixture of equal parts of 1 and 3, care being taken to equalize the dilution effect of the mixture. The result of such comparisons showed that the color in 2 was of a density greater than the purely additive effect would account for. It thus fully supported the hypothesis that arsenate can function as a catalyst to an iron catalysis—the so called "promoter effect."

These experiments have been repeated many times with uniform results, and likewise with pure iron wire to show that the iron in the nail alone catalyzed the oxidation. Attempts to obtain time curves for these experiments have been prevented by inherent difficulties. It is possible to find a suitable color standard for either the coloration produced by the arsenate and iron acting together, or for that of the color of the mixtures of solutions in which each acts alone; but the same standard will not do for both. This is due to an excess of pure purple produced when they act together and which is of itself a demonstration that the arsenate influences the action of the iron as a catalyst. By definition this is a promoter effect and corresponds to the observed effect on the respiratory enzymes of *Elodea* up to the time when it was masked by the toxic effect.

SUMMARY.

Arsenate exerts a catalytic effect on the oxidation of pyrogallol by atmospheric oxygen, on the catalytic oxidation of pyrogallol by metallic iron, and on the presumably enzymatic production of CO_2 by *Elodea canadensis*.

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